

James Baker's brave new world

The US Secretary of State is rightly cock-a-hoop about the prospects for arms control in the near future, but his vision of what follows is incomplete. But it is not too soon to be planning for the post-strategic world.

THE US Secretary of State, Mr James Baker, broke new ground on arms control in his speech in San Francisco last week. Telling how the prospects for far-reaching arms control agreements have been brightened in the past few years, he argued for paying attention now to the arrangements that will be necessary when the agreements now being negotiated have been signed and ratified. At this stage, Baker is doing little more than to think aloud, but it is radical thinking. Can he carry the rest of the US administration with him?

The list of agreements to which lawyers are putting the finishing touches is impressive. That on strategic arms has been made possible by the Soviet government's willingness to decouple the prospective agreement on strategic weapons from the Strategic Defense Initiative (SDI). That, Baker says, will be pursued within the confines of the Anti-Ballistic Missile Treaty. The objective, now, is to eliminate first-strike weapons. On conventional weapons, the superpowers seem to be moving towards an agreement based on the limitation of offensive weapons. A chemical weapons agreement is also almost within sight — the United States will destroy 80 per cent of its stocks on signature, and the Soviet Union will reduce its stocks to the same level, stocks will be reduced by a further factor of ten within eight years of a multilateral treaty and all chemical weapons will be destroyed when all states capable of making them have joined the treaty. It is a breathtaking prospect.

Is it likely to become a reality? Baker's new thinking, as *perestroika* would have it, takes the form of an open calculation of the chances. "We want *perestroika* to succeed", he says; "it would be folly indeed to miss this opportunity." But what if *perestroika* fails, and if the warp of history is rolled back? Then there will be arms control agreements in place, which the Soviet Union will be able to disown only with great difficulty, even risk. And, by implication, arms control agreements may even help *perestroika*. So far, so good.

What follows? One ingredient of the regime to come, in Baker's view, is the strategic *glasnost* provided by the arrangements for verification being written into the arms control treaties. Then, he says, there will be an opportunity also to deal with issues such as the proliferation of missile-making capacity, when the United States and the Soviet Union will be able to make common cause (as, fair play, they have done from the start on nuclear weapons).

That, he says, will also be the time to embark on the "institutionalization of a safer world".

But need it wait that long? If *perestroika* has brightened the outlook, it has also deepened some uncertainties, not only in Central Europe, but in the Middle East, the Far East and in South-East Asia. What will happen if Hungary applies to join the European Communities, or seeks to renew its old alliance with Austria? Will the problems of the Middle East spill northwards, through Iran? How will withdrawn-again China react? Where does Japan stand in this changing world? The simple answer is that nobody can tell, which is one of the reasons why present circumstances are cheerful. But that is also a good reason why the "institutionalization" of the safer world cannot be left to the United States and the Soviet Union alone. What Baker needs now, is a political agreement with Western Europe, Japan and the Soviet Union on the future exercise of their political and economic influence. Horse-trading on technology transfer is bound to be an important ingredient, mere money another. □

Diplomacy please

The US human genome project must be mounted internationally, and others must help pay for it.

THE project to sequence the human genome is running into avoidable heavy weather. That is one conclusion drawn from reports last week of the meeting between Dr James Watson, the director of the project for the National Institutes of Health (NIH), and the House of Representatives subcommittee (see *Nature* **341**, 679; 26 October 1989). One teasing issue is that of proprietary rights to whatever sequence emerges. Watson seems not greatly to have exerted himself in disabusing congressmen of the fear that spending \$3,000 million of US taxpayers' money would be a gift to the world's biotechnology industry.

In principle, this is a serious issue. Given a complete map of the human genome, commercial companies could scan it for interesting genes or arrangements of genes. Given the possibility that, 15 years from now, much more will be known of how the products of one gene regulate another, a complete sequence may yield even more subtle information about human pathology, and about the usefulness of drugs, synthetic or otherwise. But that is not



surprising. That prospect has always been part, but by no means the whole, of the justification of this important and exciting project.

So what arrangements will there be in the Human Genome Project? Even if the project is financed exclusively by agencies of the US government, it is unthinkable that its product should be regarded as a resource exclusively at the disposal of US biotechnology and pharmaceutical industries. Restrictions on access necessary to secure that would undermine the enthusiasm of sequencers in the scientific community, would conflict with the institutional obligations (to publish) of many of those participating and would rid the project of its other justifications, not least its bearing on the evolution of gene systems and even whole species.

But the Human Genome Project is not a project just to produce a single sequence, but one to understand both how the human genome functions and its relationships with the genomes of other species. If US biotechnology considers a human genome sequence would be worthwhile, it should get itself an anti-trust waiver, club together and produce one, with whatever confidentiality it considers appropriate. The project the US Congress is being asked to support is a different project, one that is worthwhile in its own right, but which may bring incidental commercial benefits as well. It should be an international project, intellectually as well as financially.

Watson's show of xenophobia last week was almost certainly directed at Japan, whose government has been slow in agreeing to contribute \$300,000 to HUGO, the embryo organization designed as a framework for sharing the labour. Unless that can be made to function, the US Congress should keep its money in its taxpayers' pockets.

Catalogue of errors

The British government needs to learn from the way it has damaged its own research enterprise.

GOVERNMENTS everywhere have well-trying ways of avoiding unwelcome advice. One is to respond energetically to the least unpalatable bits and to ignore the rest. That is how the British government responded, in 1982, to the trenchant and prophetic complaints of the House of Lords Select Committee on Science and Technology about the neglect of British science. Radical proposals, cabinet representation for research for example, were swept aside, but the government did promise an annual review of spending on research in Britain. But even that seemingly innocuous promise has now back-fired, for the government now finds itself recording the consequences of its own failings (see page 3).

Especially because last week's abrupt resignation of the chancellor of the exchequer may induce an unwonted inclination to reflection, the British government should now ask how its good intentions and the seeming logic of its policies can have so damaged the scientific enterprise.

There is no doubt of the government's commitment, when elected 10 years ago, to the view that science and technology are the sources of innovation and are thus public goods. But it was frustrated that seemingly high public spending on research and development yielded so little commercial benefit. It was also moved by animus against British universities, whose academics were judged to include too many genteel layabouts.

Three strands of policy have done the damage. The enforced monetary restraint between 1979 and 1983 damaged the research budgets of public agencies, more than offsetting the prime minister's promise that research council spending would be "protected", but the effects have since been continued by the indolence of ministries towards research and, more recently, by the decision that "near-market" projects should be supported by industry, not the public purse. That might make sense if there were an objective measure of how near is "near", but this has been but part of a long struggle to put the cart before the horse. The government believes that industry, not taxpayers, should pay for applied research, which would be correct in an ideal world. In the real world, the government cut back, but industry only barely took up the slack.

Second, despite saying that governments should not interfere, this government has been a veritable busybody, urging research agencies to spend funds on developments in technology of all kinds, as well as creating circumstances, in which funds intended for research had to be spent on reorganization and the early retirement of researchers. This journal's frequently repeated view that the research councils could have responded more effectively does not excuse the pressure, or its shortsightedness.

Third, the government has consistently underestimated the importance of higher education and training. Its suspicion of the universities may explain the indifference of its early period, when it hoped that student numbers would decline, but the shortages of novel skills apparent throughout the decade should have moved it in the other direction. There is no knowing whether its conversion this year to increased enrolments will be in time or whether it will be feasible. Meanwhile, it is no surprise that the proportion of young graduates in science and engineering (a stagnant total) seeking careers in science and technology is declining. Why enter a demoralized and badly paid profession when there is a better life elsewhere?

The consistent underlying error is the British government's failure to appreciate the gap between intentions and reality. In its treatment of research, as elsewhere, it has been seeking to effect a revolution — less direct support from government (as in Japan), more creative relationships between academic research and industry (as in the United States) and a greater responsibility on the part of students for their own educations, as well as a greater sense of its importance. The objectives have been clear. But having identified them, the government has adjusted its own behaviour and its budgets to conform, forgetting that the compensating behaviour required of the others has not usually been forthcoming. □

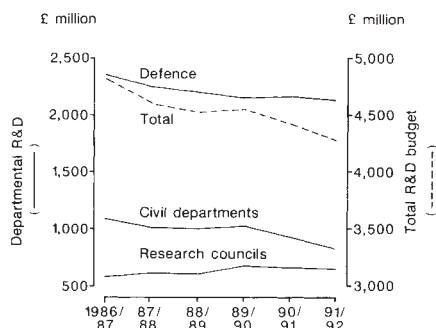
Decline in UK research spending continues

- 30 per cent fall over five years
- Britain lags behind competitors

London

BRITISH government spending on research and development is projected to fall by up to £250 million over the next three years, according to the latest review by the Cabinet Office Science and Technology Unit. The report also shows that the government is still spending proportionately less on research and development than competitor countries.

The report, *The 1989 Annual Review of Government Funded Research and Development*, predicts that total government expenditure, in cash terms, will fall from £4,616 million in 1987–88 to £4,282



Research and development expenditure by departments, measured in real terms.

million in 1991–92. In real terms, using the report's GDP (Gross Domestic Product) deflator, this represents a fall of 30 per cent in the government's contribution. The total expenditure figure is split into three blocks: the funds allocated by the Ministry of Defence, other ministries, and the five research councils and the Universities Funding Council (previously the University Grants Committee).

The government last year announced plans to switch funds from "near-market" research to basic research. This shift is reflected in the slashing of funds to the ministries. For example, between 1987–88 and 1991–92, the Ministry of Agriculture, Fisheries and Food's research budget will fall from £113.8 million to £93 million, while the Department of Trade and Industry's expenditure will fall from £324 million to £201 million over the same period.

The money saved by cuts in applied research is to be ploughed back into basic research in line with the advice of the Advisory Board for the Research Councils (ABRC) to the Secretary of State for Education and Science. In real terms, the sums available are £13.3 million in 1990–91 and £27.7 million in 1991–92.

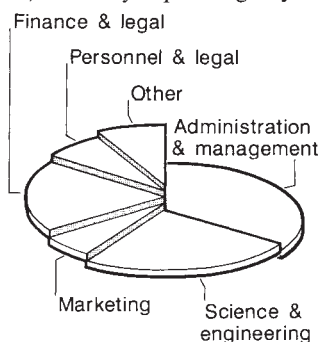
There is also to be a transfer of funds

from the Universities Funding Council to the research councils. Thus the government's estimate of universities' spending on research out of their general subvention, was £760 million last year, but will fall to £668 million in 1991–92. The overall effect is that, although what is called the Science Budget — the funds spent by the five research councils — will have increased, the total amount of government spending on science will have declined.

Another section of the report shows that Britain is still lagging behind competitors in the proportion of GDP spent on research and development. In 1987, government spending on civil research and development amounted to 0.58 per cent of GDP, compared with 0.91 per cent in France and 0.96 per cent in West Germany. On defence, Britain spent 0.54 per cent whereas the United States spent 0.88 per cent.

The figures also show that, although Britain is getting richer, smaller proportions of GDP are being allocated to research and development each year. Between 1982 and 1987, government spending on civil research and development declined from 0.70 per cent of GDP to only 0.58 per cent. Over the same period, the figure for France rose from 0.84 per cent to 0.91 per cent.

The decline in government support for research has been partly offset by increases in research support from other sources, notably spending by British



First destination of UK science graduates in 1987.

industry and the activities of international research agencies, especially the European Commission. (The report says that British applicants routinely win more than 20 per cent of the total sums awarded under European Communities' contracts, larger than Britain's 18.9 per cent contribution to the total cost of the Commission's programme.)

On this basis, the report says that the total value of research and development in Britain financed by all sources amounted to 2.3 per cent of GDP in 1987, the same proportion as four years earlier. During the same period, the corresponding proportions in West Germany increased from 2.5 per cent to 2.8 per cent and, in France, from 2.1 per cent to 2.3 per cent. In Italy over the same period, research spending increased from 1.0 to 1.3 per cent of GDP.

The fall in British government support for research and development is matched by a decline in graduate interest in science careers. The report notes that many science graduates are drifting away from careers in research, preferring service industries. More of the 11,835 science graduates in 1987 chose a job in administration and management than in any other field, including science and engineering.

Only 3,037 graduates chose to stay in research, whereas 4,010 opted for administration and management and 2,125 for legal and financial professions. The popularity of careers in science and engineering among engineers was significantly higher, with 6,412 out of 8,911 graduates staying in the profession.

Ben Webb

AIDS RESEARCH

Have grant, will travel

Bonn

IN an attempt to bring some of its best young biologists into AIDS research, West Germany has announced a lucrative fellowship programme. Beginning in 1990, as many as 50 candidates a year will be awarded travel grants and salary (but no automatic research support) to spend two years in a foreign laboratory. Three more years of support will follow after the candidate returns to West Germany.

The programme, which will initially last five years, will be administered by the German Cancer Research Centre (DKFZ) in Heidelberg, with the funds being provided by the West German Research Ministry (BMFT). About DM40 million (\$21 million) has been set aside.

Despite the policy of Research Minister Heinz Riesenhuber that no good project should fail for lack of support, the number of suitable applicants for money so far made available for work on AIDS has not fulfilled expectations. Policy-makers are aware that there are structural reasons for the gap (see *Nature* 332, 4; 1989) but are wary of setting up a research centre for AIDS for fear that it would not be flexible enough if research priorities should change.

By reaching out to the younger generation — applicants must be no older than 35 and must have completed a doctorate less than five years before applying — the Research Ministry is trying to encourage future research groups.

Steven Dickman

Officials try to allay fears

Washington

HEALTH agencies in the United States and Europe are trying hard to put to rest fears of a connection between the use of human insulin and the deaths of several insulin-dependent diabetics.

The issue, which has caused some alarm in British medical circles in recent weeks, is based upon an apparent increase in the number of deaths, due to "hypoglycaemic unawareness", in diabetics who have switched from animal-derived to human insulin.

Hypoglycaemia, or insulin reaction, strikes diabetics when the level of insulin in their blood is too high relative to their blood sugar level. The hypoglycaemic reaction is normally preceded by numerous physical symptoms such as hunger, sweating and poor coordination, and is reversed by consuming sugar. But anecdotal reports in Britain have suggested that in some diabetics who have switched from animal-derived to human insulin, the warning symptoms of a hypoglycaemic reaction are much less noticeable. This phenomenon has been linked to 17 deaths in Britain in the past year.

No scientifically established link exists between hypoglycaemic unawareness and use of human insulin, but enough concern has already been generated in Britain to have spurred further investigation. As in the United States, most of Britain's 200,000 diabetics have now switched from animal-derived to human insulin. Although some human insulin is manufactured by conversion from animal sources, much of it is synthesized using recombinant DNA technology, a fact that may well have inflamed the fears; human insulin is one of only a few genetically engineered products in wide use.

The UK Committee on the Safety of Medicines announced last month that with the help of the British Diabetic Association (BDA) it would investigate the reports of hypoglycaemic unawareness. In a recent UK survey, 6 per cent of 302 randomly selected diabetics reported diminished awareness of hypoglycaemia after switching from animal to human insulin. An earlier Swiss study, from 1987, reported diminished awareness in more than one-third of the 176 patients interviewed. Meanwhile, the BDA is said to have received some 60 unsolicited letters from patients complaining of hypoglycaemia with human insulin, sometimes with altered awareness.

With concern spreading overseas, the American Diabetes Association last week issued a statement urging people with diabetes "not to overreact". "There are no scientific studies confirming these initial reports", the statement stresses, adding that, given the complexity of mechanisms

that warn the body of hypoglycaemia, it "is very unlikely to be due simply to the type of insulin used." Dr Robert E. Silverman, chief of the diabetes programme branch of the US National Institutes of Health (NIH), says that hypoglycaemic unawareness does not seem to be a problem in the United States. Silverman's group is sponsoring a comprehensive long-term clinical study of diabetes, involving some 1,400 diabetic subjects, and so far an increase in hypoglycaemic unawareness "hasn't jumped out of our data", although the issue is not a specific focus of the study.

Silverman is confident that if hypoglycaemic unawareness were a problem among US diabetics, indications would have shown up through the NIH study or in the adverse drug reaction programme sponsored by the US Food and Drug Administration. Although he does not dispute the UK findings, Silverman has

WEATHER FORECASTING

Monsoon prediction model

New Delhi

ACCURATE predictions of the behaviour of the Indian monsoon for the past two years have bestowed a new confidence on the scientists of the Indian Meteorological Department (IMD) and their new long-range forecasting model. Monsoon forecasts have great economic and even political value; the amount of rain that falls from June to September affects not only the agricultural success of large parts of the country but also, as a consequence, their political stability. Earlier efforts to predict monsoon behaviour have, however, been largely fruitless.

The IMD now claims it has a qualitative model good enough to be a useful tool in decision-making. Meteorologists say they can predict, two months in advance, not only whether the monsoon will be good or bad but whether total rainfall will be more or less than the normal 88 centimetres. The model is a simplistic one, based on isolated and empirical discoveries of links between a number of regional and global meteorological and oceanic parameters and the monsoon circulation. Fifteen such parameters have been identified as having a historical relationship with monsoon rainfall. There seems to be a link, for example, between the strength of the El Niño phenomenon off the South American coast and the monsoon rainfall in the following year. And extensive Himalayan snow cover in winter, which increases albedo and leads to less continental heating, betokens a poor monsoon that summer.

From a study of monsoon rainfall

reservations about them because they are based upon "retrospective reporting by patients of something that happened in the past". But he says that animal-derived and human insulin are known to have different kinetics of absorption into the bloodstream, and that close monitoring is merited when patients alter their treatment regimens.

Edward West, a spokesperson for Eli Lilly and Co., the largest manufacturer and distributor of human insulin in the United States, warns against what he calls "a great deal of misinterpretation" over the issue, and adds that none of the reported deaths have occurred in patients who were taking insulin manufactured by his company. Nevertheless, West says that his company already incorporated warnings about the potential for altered awareness of hypoglycaemia in its patient literature on human insulin. In response to a request by the British Licensing Authority, all insulin manufacturers in Britain are said to have made the change in their literature as well. **Seth Shulman**

between 1951 and 1987, IMD meteorologists argue that India has a normal monsoon whenever at least nine of the 15 parameters are favourable. By contrast, 1965 was a year of extreme drought and none of the parameters was favourable that year, whereas in 1955, which saw the heaviest monsoon of this century, only one parameter was unfavourable.

Despite the empirical nature of this model, Vasant Gowariker, secretary to the Department of Science and Technology, says IMD is "quite convinced" that a qualitative prediction of the monsoon can be made simply on the basis of how many of the 15 parameters are favourable. The model was first put to use in 1988, when 13 of the 15 parameters indicated a good monsoon. In May that year, a month before the onset of the monsoon, IMD predicted a monsoon with total rainfall "on the positive side of normal"; the rainfall turned out to be 19 per cent above normal. The following year IMD predicted, this time two months in advance, another good monsoon and, at 30 September 1989, the total rainfall stood at 1.4 per cent above normal. Correct forecasts for two successive years, claims Gowariker, have "validated the utility of our model as a decision making tool". IMD scientists hope to derive quantitative predictions of the monsoon rainfall by weighting the 15 parameters differently. Like any statistical technique, Gowariker says, these models have limitations, "but they are the best available to make long-range forecasts of the monsoon."

K.S. Jayaraman

NUCLEAR CLEAN-UP

Cynics still unsatisfied

Washington

THE US Department of Energy (DoE), under the leadership of new Energy Secretary James D. Watkins, has attracted public attention and a good deal of praise for its announced determination to clean up the nation's contaminated nuclear-weapons production facilities. But even before it has properly begun, DoE's much publicized five-year environmental plan has come under attack, from private organizations and other government agencies, for being over-ambitious, impractical and even misleading.

Senate hearings on the clean-up plan are not expected until later this month and the period for public comment has not ended, but there is already controversy. Officials from the National Academy of Sciences (NAS) and the Environmental Protection Agency (EPA), as well as a variety of independent technical experts, have set out harsh criticisms of the voluminous plan, which was announced in August and unveiled officially in September (see *Nature* 340, 416; 1989).

Particularly pointed are comments from the privately funded Natural Resources Defense Council (NRDC), which claims that DoE's new accounting procedures exaggerate the true amount allocated to the environmental clean-up. The group claims that the advertised \$19,000-million cost of the five-year plan includes basic operating and maintenance functions at the weapons-production facilities, costs which DoE is obliged to spend anyway and which do not represent any new commitment of money to clean-up operations.

According to Jim Werner of NRDC, the DoE's plan includes routine maintenance items, such as replacing waterline valves, in its budget. Numerous similar examples, he says, argue that a large portion of the plan's budget has "nothing remotely to do with environmental restoration". Branding the situation an "old accounting trick", Werner says that his analysis shows that as much as 60 per cent of the DoE plan's financing is merely money shifted from existing budget categories.

Meanwhile, other agencies have criticised the plan's lack of detail. The NAS Board on Radioactive Waste Management, for example, criticizes DoE for not ordering the contaminated sites according to some sort of priority system. Without such a ranking system, the NAS's review declares, the plan lacks "focused information on the specific, near-term actions that DoE plans to initiate" and risks "promising more than can be delivered".

Senior EPA officials have also objected formally that the plan "lacks specificity and depth". In addition, these officials

complain that the DoE has not placed a high enough priority on compliance with current environmental laws in the work that it is doing at present at its contaminated facilities. And finally, the DoE has received sharp criticism for not allocating specific funds for research and development activities despite their stated emphasis on an "aggressive R&D program" devoted to the development of "environmental restoration, treatment, and disposal technologies".

Criticism of the DoE clean-up plan comes despite the overall high marks given to the Energy Secretary by most observers. Since he assumed command at the Energy Department, Watkins has been a highly visible and unstinting advocate of everything from increased science education to keeping alive the Shoreham nuclear power plant in Long Island, New York. Notably, Watkins is given credit even by his critics for his repeated and forceful public assurances that the issues of hazardous and radioactive contamination at the DoE's environmentally plagued facilities will henceforth receive the agency's highest priority.

The NAS report, for example, praises what it terms the "much publicised 'change of culture'" at DoE; this refers to an early speech by Watkins in which he admitted that DoE administrators had in the past tended to evade their responsibilities and to let environmental concerns go unanswered.

Many other commentators similarly endorse Watkins' actions, but some are more cynical. James Beard, of the Washington-based Environmental Policy Institute, for instance, acknowledges that there is a new attitude at DoE, but says it is, so far, "more a change in public relations than policy".

Defending the clean-up plan, Watkins has said that he intends it to be a "living document" subject to periodic review and amendment. This sentiment is reiterated by DoE officials, who claim that the plan must be viewed only as a broad "roadmap" for the first stages of a clean-up programme that DoE believes will take 30 years.

Nevertheless, the extent of the current controversy indicates a widespread scepticism about DoE's management record, as well as concern over the enormous federal resources that will be needed to clean up the 40-years' worth of contamination. One influential congressional staff member underlined the crucial nature of the current debate. "The last thing anyone wants", he says, "is to participate in hearings several years from now about how DoE wasted billions of tax dollars in a flawed clean-up program".

Seth Shulman

ALASKAN OIL SPILL

Suit and countersuit for Alaska and Exxon

San Francisco

RELATIONS between the state of Alaska and the Exxon Corporation, already strained in the aftermath of the oil spill last March in Prince William Sound (see *Nature* 341, 174; 1989) reached a new level of tension last week when the oil company announced a lawsuit against the state, claiming that Alaskan officials had interfered with the company's clean-up efforts.

The lawsuit, filed in the Superior Court of Alaska and seeking unspecified damages, drew an angry response. "They're just trying to rewrite history for legal reasons", asserted Ernest Piper, special assistant to the governor. He said the claim was "so contrary to fact that nobody even considered it an issue".

Exxon's complaint is actually a counterclaim to a suit already brought against the company by Alaska, which is seeking unspecified punitive and compensatory damages for alleged violations of state, common and general maritime laws.

The counterclaim focuses on an oil-spill contingency plan approved by the state for what is known as Zone 1, the central portion of the sound where most of the 11 million gallons of crude oil were spilled from the Exxon tanker *Valdez* when it struck a reef.

Both sides agree that the plan allowed chemical dispersants to be used at the discretion of the US Coast Guard alone, without prior approval from the state. But Exxon claims that the state nevertheless made known its opposition to the chemicals, which break down oil into microscopic droplets that are easily dispersed by normal tide motions, to the extent that the Coast Guard delayed granting permission for their use.

As a result, says Exxon, most of the oil remained untreated in Zone 1 for two days. By the time approval came, a violent storm had blown the slick out of the area. "It was a foreseeable consequence of the state's preventing the timely use of dispersants that much more oil would wash onto beaches, shorelines and islands and into intertidal and estuarine areas", Exxon said in the suit, which seeks reimbursement from Alaska for the part of the clean-up costs and damage claims caused by the state's alleged actions.

Piper responded that the state did not interfere at all in the clean-up, and that the Coast Guard delayed its approval solely because of unsuitable weather conditions. He said that dispersants work only in agitated seas, and that for the two days in question the seas were so flat and calm it would have been pointless to use them. "The state in no way delayed any decision to use dispersants", he said.

Robert Buder

UNESCO on trial in Paris

Paris

THE twenty-fifth general conference of UNESCO (the United Nations Educational, Scientific and Cultural Organisation), now in progress, will be a testing occasion for the organization and for its director-general, Federico Mayor.

The agenda for the month-long (17 October – 16 November) meeting at the Paris headquarters includes Mayor's medium-term plan for 1990–95 and the budget for the next two years. But also in the balance are his efforts over the past two years to carry out reforms designed to persuade the United States, Great Britain and Singapore to rejoin the organization; they left in December 1984 professing unhappiness with UNESCO's effectiveness and political direction.

Since his election two years ago (see *Nature* 329, 659; 1987), Mayor's liberal style has, for many, been a welcome contrast with that of his autocratic predecessor, Amadou Mahtar M'Bow. He has also

RAINFOREST DESTRUCTION

Unions turn green in Australia

Sydney

CONCERN over the fate of South-East Asian rainforest has led Australian labour unions to take an interest in ecology. The Building Workers Industrial Union (BWIU) for the state of Victoria, noting the daily loss of 11 square kilometres of Malaysian forest, has banned the use of imported rainforest timber. According to the BWIU, more than one-third of such wood is used to build forms for concrete-pouring, and then discarded.

Domestic timber should be used instead, the union believes, which would provide more jobs for Australians, save endangered species of Asian trees and help native groups such as the Penan tribe of Malaysia whose traditional forest habitat is only a year away from destruction.

The BWIU ban is supported by the Australian Council of Trade Unions, which has announced its intention to end the importation of timber from threatened rainforest areas. The Waterside Workers Federation and the Seamen's Union have begun a series of 24-hour actions to prevent the unloading of timber from ships docking in Australian ports.

But according to Neil Goldsmith of the Plywood Association of Australia, their actions could be counter-productive. The import of rainforest timber is a \$128-million-a-year industry, and disruptive union action could, says Goldsmith, drive up prices and cause the loss of domestic jobs.

Tania Ewing

proved himself an able diplomat by persuading the Palestine Liberation Organisation to postpone its demand for membership until the next general conference. Without this withdrawal, there would have been no hope that the United States would reconsider its membership.

But within UNESCO, Mayor has been criticized for not going far enough or fast enough in his reforms. And, despite his three meetings with the new US Assistant Secretary of State for International Organisation Affairs, John Bolton, a Congress hearing on UNESCO in September, at which non-governmental organizations and the private sector were invited to testify, made it apparent that Mayor has still not convinced some sectors of the US administration.

At the hearing, Frank Press, former director of the National Science Foundation, Leonard Sussman, author of a recent book on press freedom and Dr Mitchell of the American Council on Education gave testimony on UNESCO which, according to an observer at the hearing, emphasized its potential future role in international cooperation in science. They implied that the United States could no longer afford to stay outside the organization despite its faults. But Bolton, a Bush appointee who came from the hard-nosed Heritage Foundation, maintained that UNESCO still has a long way to go before reforms justify a US return.

It has been difficult for Mayor to achieve real reforms on the questions that were instrumental in the US and British decisions to leave. At the heart of the quarrel was the New World Information and Communications Order (NWICO), M'Bow's attempt to protect developing countries from what was perceived as an unwelcome flood of media attention from industrialized countries. As part of its programme, NWICO effectively endorsed the right of governments to censor critical news reporting.

In his new medium-term plan, Mayor addresses this issue by falling back on the UNESCO constitution, which speaks of the organization's duty to advance the "mutual knowledge and understanding of peoples" by promoting the "free flow of ideas by word and image".

But Africa is UNESCO's main province of concern, and Mayor cannot afford to upset the African delegations; he thus refers to a need for the "balanced dissemination of information", a location that to sensitive US ears sounds like an echo of NWICO. The way out of this quarrel, says Mayor, is through the notion that the interests of developing countries can best be served by encouraging the development of endogenous communications and media.

Another cause of US irritation was the top-heavy bureaucracy. Deprived of more than a quarter of its regular income by the US walk-out, the organization was forced to cut its staff of 2,800 by 700. But, as Mayor pointed out in his opening address on 16 October, cost-cutting is incompatible with the injection of much-needed new blood. The inability to hire new staff in any quantity means that Mayor has not only had to manage a programme set up by his unpopular predecessor, but has had to do so largely with M'Bow's former staff.

The content of Mayor's medium-term plan has so far met with approval. His "priority of priorities" is to reduce the "unacceptable gap" between the richest and poorest peoples through education programmes. Next year will be International Literacy Year and UNESCO will hold a conference on Education for All in Thailand in March. Other priorities are the promotion of peace through increased cultural exchange and also of "peace with nature" through UNESCO basic science programmes, a plan whose theme is "not to mortgage the very existence of future generations".

But despite Mayor's ambition that UNESCO should "do less, but in greater depth", the organization is faced with severe financial difficulties. Taking account of a shortfall carried over from the last budgetary period, the proposed \$372.5 million regular budget for 1990–91 is effectively a decrease in real terms, and is on a level with spending in the early 1970s. This means that the programme for the next two years will be hotly contested.

Developing countries have already resisted a proposed 2.5 per cent increase in members' contributions, and plans to modernize communications equipment are likely to be postponed.

According to John Fobes, US observer at the conference on behalf of the International Social Science Council, the time is right for the United States to return. Now that the communications issue has quietened down, he says, the value of UNESCO as coordinator of international science, technology and education programmes can be appreciated.

"There will be a lot of important conferences on global issues in the next few years and it would be good if UNESCO became a quiet place for an overall look. In the next ten years, UNESCO will be rebuilt and will become a monitor, instead of a do-all and be-all. The world needs that."

Despite Bolton's luke-warm statement in Congress, some influential groups within the United States are in favour of a return. In Britain, the Foreign Office has assured Mayor that the government will review its position once the conference is over.

Peter Coles

GLOBAL CLIMATE CHANGE

Equivocation on cooperation

Washington

DESPITE President George Bush's election promises to take the lead in tackling global climate change, his administration has outraged the US Congress and environmental groups by hesitating over whether to send official representatives to an international environmental conference in the Netherlands next week. The White House Domestic Policy Council finally agreed that the president's science adviser, Allan Bromley, should accompany Bill Reilly, the head of the Environmental Protection Agency, to the conference, but, it is said, has not allowed Reilly to invite participants to another conference that would be hosted by the United States next year. A new inter-agency working group set up to develop the administration's policy on global climate change will meet this week to decide whether to support the global climate convention that will be proposed at the Netherlands conference.

The controversy hijacked a hearing of the Senate Committee on Commerce, Science, and Transportation last week whose purpose was ostensibly to consider the nominations of James Wyngaarden, the former director of the National Institutes of Health, and Thomas Ratchford to be associate directors of the Office of Science and Technology Policy (OSTP). Bromley denied that the administration was opposed to negotiating a global climate convention, but emphasized that it would undertake no action until the end of 1990, when the Intergovernmental Panel on Climate Change (IPCC) will have completed its studies. Formed under the auspices of the United Nations Environment Programme and the World Meteorological Organisation, the IPCC is reviewing the scientific evidence for global warming, the social and economic impact of possible climate changes and ways of dealing with the problem.

Senator Albert Gore (Democrat, Tennessee) said he was "extremely disappointed" with the advice Bromley was giving to Bush on this issue after Bromley's refusal to say whether he had initially been opposed to attending the conference or whether he was in favour of negotiating a convention were interpreted as indicating his opposition to both. Although earlier this year Bush signed a declaration at the G-7 economic summit that "strongly advocates" common efforts to limit emissions of carbon dioxide and other greenhouse gases and declares that a global convention on climate change is "urgently required", Bromley said that there was a difference of opinion between the administration and Congress over the definition of 'urgent'. He also said that there was "a substantial distance to go yet" before an adequate understanding of

the science of global change would be reached, and quoted a recently revised model from the UK Meteorological Office that reduced the predicted temperature increase for a given addition of carbon dioxide from 5.2 to 1.9 °C after a more sophisticated treatment of cloud behaviour was added. Bromley also emphasized that discussion so far has focused too much on "the input side of the global change equation" and that more studies are needed of the economic consequences of a convention.

Eleven environmental groups, including the Natural Resources Defense Council, the Environmental Defense Fund and the National Audubon Society, offered Bush rather different advice last week on policies to tackle global warming. In a joint declaration, they called on Bush to support a 20 per cent reduction in national carbon-dioxide emissions by the year 2000, an international convention to reduce emissions of greenhouse gases, and the development of a national climate policy. At a hearing of the House of Representatives subcommittee on human rights and international organizations, they all endorsed a bill to coordinate better federal research on climate change through the Committee on Earth Sciences of the OSTP.

Christine McGourty

IVORY TRADE

Japan agrees to total ban on imports

Tokyo

JAPAN, the world's largest importer of ivory, has at last agreed to a total ban on ivory imports, at least for the time being.

The Japanese cabinet decide on 20 October to abide by the ban on all imports implemented by the Convention on International Trade in Endangered Species (CITES) at a meeting in Lausanne, Switzerland, earlier in October. Japan at first joined South Africa, Zimbabwe and Botswana in opposing calls for a ban but then abstained at the final vote.

CITES member nations are concerned about rapidly dwindling elephant populations in Africa. But South Africa, Zimbabwe and Botswana claim that their elephant



Tonnes of ivory confiscated from poachers in Africa

ENVIRONMENT

'Hot lab' to be closed over contamination fears

San Francisco

BOWING to growing community concern over toxic contamination, and citing changing business needs, the Rocketdyne Division of the Rockwell International Corporation has decided it will shut down a nuclear laboratory, used for processing radioactive materials, at its Santa Susana Field Laboratory east of Los Angeles.

The decision, announced on 20 October and due take effect exactly a year later, will close a 30-year-old operation. Making the announcement, the company said there had been a change in federal government emphasis away from the liquid-metal fast-breeder reactors, in which Rocketdyne had done specialized contract work, towards light-water reactors.

But Rockwell has also been suffering from revelations that a US Department of Energy survey had found radioactive and chemical contamination of the soil beneath the 2,600-acre Santa Susana site, although no immediate health hazard was declared.

Rocketdyne had originally requested a 10-year extension of its operating licence from the Nuclear Regulatory Commission (NRC), but has now asked for an extension only until 20 October 1990 to allow time for the company to complete its decontamination and decommissioning plan for the facility.

Robert Buder

populations are stable or increasing due to conservation measures and wish to continue their lucrative ivory trade.

In June, the Ministry of International Trade and Industry (MITI) imposed a ban on indirect ivory imports from suppliers in such places as Hong Kong and Singapore. But ivory importers immediately began to stockpile and MITI responded with a 'temporary' total ban in September (*Nature* 341, 270; 1989).

Japan's decision to abide by the CITES ruling, rather than filing a reservation to get around the ban as several African nations plan to do, may in part be due to the fact that Tokyo will host the next CITES meeting in 1991. If imports had continued, Japan would be in a very embarrassing position.

But Cecilia Song of TRAFFIC Japan, the trade monitoring wing of the World Wildlife Fund (WWF), says that MITI and the ivory industry hope to resume trade in the near future. The Lausanne agreement allows for resumption of trade after two years if countries can show they have developed an effective strategy to protect elephant populations.

In the meantime, Japan's ivory importers have begun importing mammoth tusks from the Soviet Union.

David Swinbanks

Move raises problems

Munich

THE uncertain life of the West Berlin Academy of Sciences, which must leave its present home by July next year, seems destined to continue. Criticism from the opposition Social Democrats (SPD) in the *Land* of Hesse may prevent the beleaguered academy from moving there, despite earlier indications that Hesse would be a safe haven. The SPD has set "high hurdles", according to a spokesman, before it will accept the academy.

The academy became a source of discord in Berlin politics after it was pushed through the parliament in 1987 by the conservative Christian Democratic Union (CDU; see *Nature* 329, 659; 1987). SPD and Green Party politicians, then in the opposition, criticized the academy as a "debating club" with conservative leanings. They vowed to shut the academy once they gained power and, in May this year, the now left-wing government of West Berlin proposed a law to do just that.

With the help of his conservative coalition partners, Hesse science minister Wolfgang Gerhardt (Free Democrat) responded to the Berlin decision in June by offering to "adopt" the academy, and to give it the same financial support as it had been receiving in West Berlin — DM 8.5 million (\$4.6 million) a year. This offer needs the approval of the *Land* parliament of Hesse: debate is about to resume, and a final vote is expected in December or January.

But now academy president Horst Albach has said that he will not accept the offer unless the SPD in Hesse also agrees to support the academy. Having already been the victim of a change of government, Albach wants to avoid further uncertainty over the future of the academy.

In response, the SPD in Hesse has demanded a role for political parties in the academy's board of directors as well as strong representation by the universities and research institutes of Hesse. "The academy will be a big financial burden for Hesse", said SPD spokesman Erich Stather. "It is only logical that we want a chance to express our views. What bothers us the most is having the academy floating free of all other scientific institutions." The SPD met in private this week to discuss its position on the academy.

Will the academy be able to accept the terms demanded in Hesse and still remain independent? Academy spokesman Eberhard Vogt calls the problem a case of "Scylla and Charybdis" — the academy is trapped between two hazardous alternatives. Some members of the governing board, which manages the day-to-day affairs of the academy, oppose an industry-financed future for the academy

for fear of losing independence.

Nevertheless, the mood in the academy is optimistic. "We have been criticized by conservatives at the University of Frankfurt for being too left-wing, whereas left-wingers in Kassel have said we are too conservative", he said. "So we must be in just the right place politically now." Albach is grateful for the chance to correct the "birth defect" in the way the academy was voted into existence in West Berlin, and he has been crisscrossing Hesse in an attempt to generate support for the new version of the academy.

Ironically, if the academy were to accede to the demands of the SPD in Hesse, it would belatedly live up to the demands that the SPD in West Berlin proposed when its future was discussed in 1986-87. But this is no longer enough for the West Berlin Senate to invite the academy to stay: the Greens would oppose any such move as a violation of their coalition agreement with the SPD.

In a further irony, some activities of the West Berlin academy will probably be allowed to stay in the city if a compromise is reached in Hesse. Hesse is expected to approve the existence of such a "satellite academy" and there is even talk in the West Berlin SPD that some new academy projects would be welcome — a belated tribute to what some are calling the "academy in exile". **Steven Dickman**

BRITISH UNIVERSITIES

United stand in move to greater efficiency

London

FOUR British universities have appointed a firm of management consultants to try to make them more efficient. In the first move of its kind in higher education, the Universities of Birmingham, Leicester, Loughborough and Warwick have joined forces to maximize their use of resources to offset tighter government budgeting.

The PA Consulting Group will carry out efficiency audits of services such as residences, libraries, maintenance and catering in the four universities.

For the next three years, PA will draw up comparative studies across the four universities and will also look at each area specifically.

Professor Gerald Bernbaum, executive pro-vice-chancellor and registrar of the University of Leicester, said the universities were simply following Universities Funding Council advice to carry out an audit. He said PA would help them "share good practices" and that he was confident the study, which was at a "reasonable cost", would pay for itself in time. **Ben Webb**

New minister, old problems

Cape Town

IN a cabinet reshuffle last month, the new South African president, F.W. de Klerk, announced the return of Dr Gerrit Viljoen as Minister for National Education, with an additional appointment as Minister for Constitutional Development. Viljoen, a graduate of the Universities of Cambridge and Leiden, was professor of Greek at the University of the Witwatersrand in Johannesburg before entering politics, and had previously served as education minister from 1980 to 1984. He was subsequently made Minister of Cooperation and Development, but a year later was effectively moved to the sidelines as Minister of (African) Education and Training, reputedly because he was not a member of ex-President Botha's inner circle. But he was one of de Klerk's staunchest supporters in deposing Botha, and has been rewarded with two of the most crucial portfolios in the new cabinet.

On top of his responsibility for overseeing constitutional reform, Viljoen is faced with the task of defusing the country's growing educational crisis. In 1986, the government declared a commitment to the elimination, over a 10-year period, of racial inequalities in primary and secondary education, but earlier this year was forced to abandon that policy as economically impossible. Education already accounts for 20 per cent of budget spending in South Africa, and in the current economic climate the increases that would be needed for large-scale educational reform are unlikely to materialize. And despite its goal of educational equality, the government is fiercely committed to the provision of separate educational facilities as it regards integration of schooling as an issue too sensitive for the white electorate to accept.

Yet there are signs that this same electorate may be ready to face up to the reality of the situation. The Johannesburg High School for Girls, threatened with closure due to dwindling numbers of white students in its catchment area (which has become a *de facto* mixed area in recent years) decided earlier this year to go private instead. The state has allowed integration in private schools for the past decade, and has also given them limited state subsidies without taking away their charitable status. By allowing a state school to become independent, the government can allow integration without taking overt responsibility.

Academics in South Africa are generally optimistic about Viljoen's appointment, because of his credentials and also because during his first period in the education office universities and museums were well-treated. But he now has more difficult tasks to deal with, and fewer resources to apply to them. **Michael Cherry**

DRIFT-NET FISHING

Japan under pressure

Tokyo

JAPAN's fishermen are coming under increasing international pressure to end their use of massive drift nets on the high seas. At last week's summit in Kuala Lumpur, Commonwealth nations joined the growing chorus of countries calling for an international ban on pelagic drift-net fishing, which critics liken to 'strip-mining' the ocean.

Made of fine, nylon monofilament, drift nets can stretch across nearly 60 km of ocean and snare most fish which encounter its virtually invisible mesh to a depth of 15 metres. Designed primarily to catch squid, salmon and young tuna, drift nets are being blamed by environmentalists for the indiscriminate slaughter each year of an estimated 1 million sea birds and 200,000 marine mammals, in particular dolphins and porpoises.

New Zealand, alarmed by the possibility that Japanese and Taiwanese drift-net fleets could wipe out the South Pacific's albacore tuna fishery within the next few years, is putting the finishing touches to a resolution it will submit to the United Nations for a ban on high seas drift nets.

The United States, concerned over the 'piracy' each year of some million migrating North American salmon and steelhead trout by drift-net fleets fishing in international waters of the North Pacific, is expected to co-sponsor the resolution.

Japan, which operates 500 of the 1,300 Asian drift-net vessels known to ply the Pacific, feels talk of a ban is premature and that drift nets should be regulated as part of a comprehensive management scheme covering all fishing practices in the Pacific. With an estimated 8,000 jobs and some \$1,300 million in revenue at stake, Japan is hardly ready to give up without a fight on the issue. "We cannot accept such a radical and unilateral measure to stop our operators without more objective scientific data", said Minoru Morimoto, an official at Japan's Fisheries Agency.

By the time more conclusive data on the impact of drift nets are made available, however, it could be too late to save the South Pacific albacore fishery, according to the Forum Fisheries Agency (FFA), which represents 15 island nations in the region.

The FFA estimates the maximum sustainable catch for the entire tuna surface fishery in the region at around 10,000 tons per year, the amount caught by Japan's drift-net fleet after it expanded in size from 20 boats in the 1987-88 winter fishing season to 60 boats last year. The influx of Japanese drift-net vessels was in part spurred by rising prices for albacore and a decision by the United States last year to bar Japanese from fishing in its 200-mile exclusive economic zone.

"Since young albacore are destined for the cannery, Japanese just try to catch as much as they can without waiting for the fish to reach full size", said an official at the New Zealand Embassy in Tokyo. "This gluts the market, drives prices down and forces them to catch more. It's a vicious circle."

The region's longline fishermen, who make their living off more valuable adult albacore which generally swim at greater depth than young tuna, are already reporting a decline in the size of their catch.

If the albacore fishery is destroyed by drift-netters, an important chance for the economic development of the region may also be lost. "If the albacore fishery isn't managed properly", says Fiji's ambassador to Tokyo, Charles Walker, "we could end up with ghost towns in the South Pacific."

BIOTECHNOLOGY PATENTS

Dupont battles with Cetus

Washington

A BATTLE over the lucrative market for the widely used DNA amplification technique PCR (polymerase chain reaction) has set the US biomedical research community buzzing. When Dupont announced in August a challenge to the PCR patents owned by Cetus (see *Nature* 341, 418; 1989) it would at first give no details of the basis for the case. But Dupont's patent attorney, George Frank, now says that the company has unearthed two scientific papers describing the technique which were published more than 10 years before the research for which Cetus won its patents.

The first, a paper by H. Gobind Khorana and colleagues published in the *Journal of Molecular Biology* in 1971, describes in principle a technique that can be used to synthesize multiple copies of genes, and finishes by saying that "experiments based on these lines of thought are in progress" (see *Nature* 341, 570; 1989). In a subsequent paper published in 1974 in the *Journal of Biological Chemistry*, Khorana and Amos Panet briefly describe unpublished research from the same laboratory in which multiple copies of DNA sequences were obtained by alternate cycles of heating and cooling in the presence of primers, techniques also used in the Cetus method. Frank claims that either of these alone is sufficient to invalidate Cetus's patent, because under US patent law a patent cannot be granted if a description of the invention was published more than one year before the patent application was filed.

But the general counsel for Cetus,

While continuing to defend the use of drift nets, the Japanese government has ordered a two-thirds reduction of the South Pacific drift-net fleet for the forthcoming season. Japan has also agreed to allow a scientific observer appointed by the region to accompany a research vessel to the South Pacific this year. The Taiwanese have not yet responded to calls for a ban.

Japan has further agreed to allow the United States to track some 200 Japanese squid drift-net boats with transponders starting next June and increase scientific observations of the fleet in the mid- and North Pacific.

"Up until now, there has been little or no global management of fisheries, resources and its become a matter of urgency — regardless of whether there's drift-net fishing", concludes a US government fisheries expert. "We're entering a new era involving delicate issues of sovereignty and financial responsibility. It's not going to be easy."

Mike Shultz

Michael Ostrach, says that although the company was not aware of these papers when it filed for its patent in 1985, they will not affect the validity of Cetus's patents because they do not "teach" the reader how to perform PCR: to permit a patent to be granted, a technique must be described in sufficient detail to enable others to replicate it. If the earlier papers had described PCR, Ostrach says, researchers would have started using the technique then, instead of waiting until 1985, when the research done at Cetus was published. Frank counters that the technique was not practical in the 1970s because it was difficult to obtain primers, an explanation that Ostrach rejects.

Meanwhile, Du Pont says that it is making "substantial investments" in the development of DNA replication and detection kits for sale to researchers and that commercial distribution may begin this month. It also plans to sell thermocyclers, the instruments which automate the PCR process, and to sell PCR-based products for the diagnosis of human diseases, although Cetus representatives have told Dupont that it will not be granted a licence for diagnostic products. Dupont admits to being "in reasonable apprehension that it will be sued by Cetus" for patent infringement.

The case is likely to come to court in about a year, according to Frank, and both sides say they will have expert witnesses to support their cases. If the challenge is upheld, it will be "a cataclysmic event" for Cetus, according to Robert Cooper, a biotechnology analyst at Kidder-Peabody. **Christine McGourty**

Lax standards confirmed

London

VIKTOR Bryukhanov, director of the Chernobyl nuclear power station at the time of the 1986 disaster, has at last broken his silence and given an interview to the newspaper *Sotsialisticheskaya Industriya*. Bryukhanov, who is serving a 10-year sentence in a labour colony, had been afraid that any statement he might make would be interpreted as self-justification. "And I was guilty", he admits, "only not as guilty as the court decided." On the night of the ill-conceived experiment that ended in explosion, Bryukhanov was at home in the nearby town of Prypyat.

He was awoken, he says, by a telephone call from the head of the chemical unit who told him only that "something awful has happened — some sort of explosion". Imagining something like a ruptured steam-line, he hurried to the station, but when he saw that the top of number four block was completely missing, "my heart stood still", he says. He telephoned Moscow and tried to order an immediate evacuation, but no one would support him because it was "so firmly fixed in their minds that nothing could happen to the reactor".

Bryukhanov's account confirms the picture which emerged at the post-accident review conference held by the International Atomic Energy Agency of a naive faith in the safety of nuclear power coupled with a neglect of the very rules and procedures on which safety depended. But as director of the Chernobyl power plant from its inception, he is able to reveal the background to this atmosphere of rule-breaking. Starting with a green-field site, he had to oversee the construction not only of the power station itself but also temporary accommodation and later a town for the site-workers. He found himself coping with broken heating systems and failing supplies of fresh vegetables and, unlike the directors of manufacturing plants, he had nothing he could barter to get consumer goods for his workers.

Bryukhanov maintains that without rule-breaking, the Chernobyl station would never have been completed. Electric cables, for example, should have had fire-resistant claddings, but none was available and ordinary cables were used instead. He says he was worried about the situation, but knew that if he made a fuss he would simply be replaced by a new and more amenable director. "It is not within the power of one man to overthrow a system of economic-management relationships which has built up over decades. And he should not be held guilty if in the end he becomes the slave of that system."

But Bryukhanov was found guilty while

others in higher places escaped to honourable retirements. Long before the Chernobyl accident, he says, scientists from the Kurchatov Nuclear Energy Institute warned Academician Anatolii Aleksandrov (president of the Academy of Sciences and a leading supporter of nuclear power) of design faults in the RBMK reactor, but nothing was done.

After the accident, the safety systems were tightened up, making it impossible to withdraw many control rods (as was done at Chernobyl) and considerably reducing the automatic shut-down time. Yet Bryukhanov says he is not certain that events similar to those of Chernobyl will not be repeated elsewhere.

CLINICAL RESEARCH

Network of centres better than just one

London

PLANS by the UK Medical Research Council (MRC) to establish a national clinical research centre have been rejected by the Advisory Board for the Research Councils (ABRC), which favours instead a network of provincial research centres. These, says the ABRC, will meet the desired aims of improving the quality of science and the standards of medical training and hospital practice, but will be more cost-effective than the MRC's strategy.

Although Dr David Rees, MRC secretary, welcomed the ABRC's support for the clinical research initiative, he was disappointed that it had not approved the idea of a single centre of excellence. The proposed centre would have been located at Hammersmith Hospital, in association with the Royal Postgraduate Medical School, at an initial estimated cost of £100 million. That figure was later scaled down to £50 million after the ABRC requested that funds be released for other leading clinical research centres in the United Kingdom.

The ABRC recommends that the Hammersmith centre should be fostered, and that the Clinical Research Centre (CRC) at Northwick Park should be closed so that resources can be redirected to the provincial centres. Sir David Phillips, ABRC chairman, in a letter to John MacGregor, Secretary of State for Education and Sciences, says there is a strong case for "enhancing and sustaining" clinical research at the RPMS through an infusion of basic biomedical research, but adds that the ABRC does not favour a single national centre, preferring a "distributed approach" with "phased developments" at a number of provincial centres. Phillips concludes that this programme would cost "significantly less" than the single-centre option.

Bryukhanov has been heavily criticized since the accident, and the attacks continue. Only last summer, the weekly *Argumenty i Fakty* said that the three operators who carried out the ill-fated experiment on the reactor did not know that pulling out too many rods could cause an explosion. Their superiors did know, the paper said, but they were safely asleep at home.

Whether Bryukhanov did know the full danger and could have stopped the experiment is unclear. He portrays himself as a man in an impossible job, who was made the scapegoat when disaster struck. In the words of Georgii Kopchinskii, head of the Department of Atomic Energy of the Bureau of the Council of Ministers of the USSR, the court "did not judge the man, but the job".

Vera Rich

MacGregor said he was "naturally pleased" that the ABRC's preference on merit was for the cheaper approach. Dr James Scott, of the CRC, said the MRC had "gone for the stars" in asking for backing in the national research centre initiative and that it should not be too disappointed that it had not got complete support. He said the CRC had not been the success it should have been and that clinical research needed to "pull its socks up". Ben Webb

AIDS

AZT for children in extended US trial

Washington

THE US Food and Drug Administration (FDA) last week approved the use of the anti-AIDS drug AZT (zidovudine) to treat children.

Under the terms of a treatment IND (investigational new drug) programme, AZT will be provided free of charge by its manufacturer, Burroughs Wellcome, not only for children showing symptoms of AIDS but also for those lacking symptoms but in the advanced stages of infection with HIV (human immunodeficiency virus).

Two months ago, on the basis of trial results demonstrating that AZT slowed the progress of HIV infection, the drug was made available to asymptomatic but infected adults as well as those with overt AIDS. The new extension is also based on trial results: a study of more than 200 children has shown that AZT can both relieve symptoms and prolong survival. What had held back use of the drug for children was the fear that side-effects could be more severe in children than in adults, but the study results so far indicate that side-effects are not qualitatively different in children.

David Lindley

For unto him that hath . . .

Virginia Trimble

The concept of P-selection, by which graduates of the more prestigious (P) universities tend to publish longer than those from other (NP) universities, is herewith introduced into the literature.

If you really want a career in scientific research, then get your PhD from the most prestigious university that will take you. This conclusion (which should surprise very few) follows from data collected for the Astronomy and Astrophysics Survey Committee, now in the process of preparing a report to the US National Academy of Sciences on Astronomy and Astrophysics for the 1990s.

To be precise, astronomers who received their doctoral degrees from a university that ranks repeatedly among the 'top three' American astronomical PhD programmes¹ continue to publish in the field about twice as long as those with degrees from a university that ranks in the 'second ten'. In addition, they are about twice as likely to have current jobs that primarily involve research and/or advanced teaching at observatories or PhD-granting universities.

One can imagine a variety of reasons for the difference. The more prestigious institution may genuinely provide a better education, or it may skim the cream from the available pool of entering students. Or its name may serve as a lubricant to smooth the paths to research grants, acceptance of papers and good jobs for its alumni, at least early in their careers. The data say nothing whatever about the relative importance of these or other causes for the phenomenon, though I am privately of the opinion that the third factor is not negligible.

The sample populations include 106 recipients of PhDs between 1952 and 1988 from the high-ranking institution (hereafter P) and 94 recipients of PhDs between 1966 and 1988 from the lower-ranking one (NP hereafter). Figure 1 shows their publication histories. The horizontal axis is number of years since receipt of doctoral degree. The vertical axis is the fraction of all astronomers who had had their degrees for at least N years who were still publishing papers listed in the semi-annual issues of *Astronomy and Astrophysics Abstracts*² at least N years after their degrees. Upper points pertain to P and lower ones to NP, and the differences are large enough to require no words to guide the eye.

The doctoral programme at P is the older one, and it might be hypothesized that the difference shown in Fig. 1 is a result of good jobs being easier to get in the past (back when our towers were made of real ivory rather than plastic and all our students were above average). As a test of

this hypothesis, Fig. 2 shows the publication histories of post-1966 degree recipients from the two universities (85 from P, 94 from NP). Indeed the difference between the two is smaller than in Fig. 1, but the P astronomers are still about twice as likely to continue to publish for more

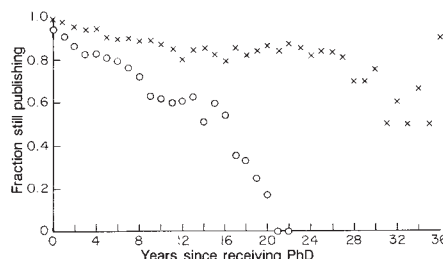


FIG. 1 Fraction of astronomers who have held PhDs for at least N years who are still publishing research papers N years after their degrees. Upper points = people from the prestigious university, P; lower points = people from the non-prestigious university, NP. Neither set of points starts out a unity because a few people from each place never published anything. Numbers drop below 10 people at 18 years for NP and 28 years for P.

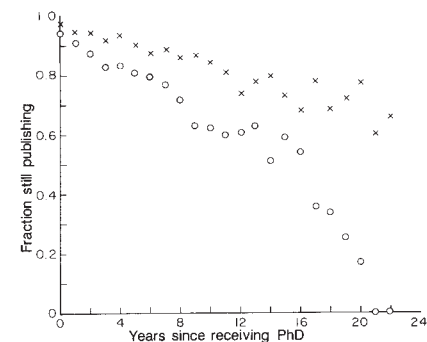


FIG. 2 Fraction of post-1966 degree recipients who are still publishing in astronomy as a function of number of years since the PhD. Upper points = P alumni; lower points = NP alumni. Numbers drop below 10 people between 18 and 19 years post-PhD for both.

than 18 years (when both samples drop below 10 members) than are the NP ones.

Current employment patterns of the two groups are also significantly different. There are members of each group in each of nine categories: (1) astronomy research and teaching at observatories and PhD-granting universities; (2) astronomy research at government and industrial laboratories (like Los Alamos National Laboratory and AT&T Bell Laboratories); (3) research in other sciences; (4) scientific support services (data processing, hard-

ware and software development); (5) industrial employment; (6) government employment (generally science administration); (7) teaching at places with no PhD programme in astronomy; (8) employment outside science; and (9) lost to follow-up and not publishing in astronomy (two from P, three from NP).

The most striking difference is that 65 per cent of the P astronomers, but only 32 per cent of the NP ones, have jobs in category 1. A large majority of incoming graduate students at both places describe their career goals as research and advanced teaching in the field. Most graduate programmes concur that their primary purpose is the production of the next generation of researchers. Thus the P astronomers can be described as more successful than the NP ones by their own standards.

Life being somehow easier in the past is not the primary cause. An artificial sample of P astronomers, age-matched to the NP ones, has 66 per cent of its members in the most desirable category of employment, just the same as for the total P sample. Curiously, this does mean that things are getting worse. People continue to diffuse out of the astronomical research community over their entire careers. But they almost never come back (though there is diffusion into the field of PhDs from physics and other sciences). No one in either sample resumed publishing after a hiatus of as long as five years. And the few returns to jobs in categories 1 and 2 occurred only when the excursions had been brief ones to positions in science administration. Thus we can expect that, 10 or 20 years from now, the fraction of the 1966–88 P astronomers still in research and advanced teaching will be less than 65 per cent and so smaller than the fraction of the total P sample now engaged in these activities.

The moral of the story is thus two-fold: it still pays to go to the most prestigious graduate institution you possibly can, but the gold paving, even on the streets of P, is not as thick as it used to be. □

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1. Gourman, J. *The Gourman Report* (National Education Standards, Los Angeles, 1987).

2. Esser, U. et al. (eds) *Astronomy and Astrophysics Abstracts*, Vol. 45 and earlier volumes (Springer, Heidelberg, 1989).

Criminalization of drugs

SIR—Your leading article "Bush declares war on drugs" (*Nature* **341**, 1; 1989) is right in noting that if drug-related crime is to be totally eliminated, all drugs would have to be decriminalized, but it must be hoped that neither the United States, nor any other country for that matter, ends up decriminalizing narcotics or illicit drug use altogether.

You ask "would not a society in which narcotics were accessible that did not destroy itself by addiction be a stronger society and perhaps even kinder and gentler as well?". Problem drug-users have frequently told me, in the ten years in which I have worked with them, that "heroin blanks you out, it makes you emotionally distant". Many other drugs have even nastier and more antisocial effects. In the affluent West, the desire to achieve self-realization is common. Unfortunately, because of large-scale pharmaceutical drug use, it has become a drug-focused society. To be realistic, heroin and other euphoric and mind-altering drugs, whose use can at first be pleasurable, would be used on a wide scale if they were easily accessible. Would we want large sections of the population wandering around emotionally isolated and cut off from meaningful relations with others? Such a society would hardly be "kinder or gentler".

Clearly some degree of criminalization is necessary. But what is needed is a sensible policy suited to the real needs of containing the drug problem. For example, heroin, cocaine and crack are this decade's fashion. Designer drugs will be the major threat in the future. Should there not be international laws outlawing and controlling the illicit manufacturing of all synthetic drugs before they become a fashion?

Further, the British did not, as the author claimed, give up prescribing narcotics some 20 years ago because of the over-prescribing of one doctor. Rather, in the 15 years after the drug dependency clinics were set up in 1968, their consultant psychiatrist directors generally found that such long-term prescribing was medically harmful. Also, it generally did not help addicts to lead normal lives or keep them from crime. Even so, a small number of doctors continued to maintain addicts. Recently, in a move to combat AIDS, their numbers have increased. Most, however, offer only short-term treatment with reducing doses and aim to wean their patients off drugs as soon as possible.

Liberal prescribers such as Trebach argue that the British system of treatment was the reason why, until the 1970s, Britain had only a small drug problem. But, as I have indicated elsewhere, it was precisely because numbers were small that such a policy could be pursued. When the size of

the addict population rapidly escalated in the 1980s, treatment and prescribing policies were ineffective in controlling the size of the addict population because heroin was cheap and easily available on the street. While illicit drugs remain easily available on the street, many of the addict population will, for a number of reasons, prefer not to seek maintenance prescriptions, but seek to buy illicitly — if for example, they are only recreational users, or they hate authority and the restrictions imposed by drug dependency units or prefer the excitement of the illegal subculture. And the drug market is organized in so complex a way that it would stimulate a demand on the street for their pharmaceutical drugs. Instead, large-scale liberal prescribing would increase the size of the illicit market, for it would lead to an extensive pharmaceutical blackmarket fed from the overspill from liberal prescribing.

It would be a very foolish policy-maker indeed nowadays who confused 'treatment' with drug control policy or containment.

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Collision course

SIR—The race between the Stanford Linear Collider and the Large Electron-Positron Collider at CERN, the high-energy physics laboratory near Geneva, to be the first to elucidate the properties of the Z^0 particle raises fundamental questions about the purpose of particular scientific research projects in an era of increasing costs and declining resources. It was known from the beginning that the CERN machine would be able to produce Z^0 s in much larger numbers than the Stanford machine. Thus, there was never an expectation that Stanford could produce data that would not eventually be available from CERN. The only question was whether Stanford could produce some of these data first. If some properties of the Z^0 had become known a few months earlier because of the Stanford experiment, it would have had absolutely no effect on scientific progress. It would, however, have been of significant advantage to the careers and reputations of the scientists involved in the Stanford experiment.

When the cost of a single machine such as the Superconducting Super Collider can approach the gross national product of a small developing country, we should question whether the taxpayers should be continuously called upon to support this kind of pointless competition and duplication of effort. If CERN, the United

States, the Soviet Union and Japan cannot get together to fund and build a single high-energy physics machine, we should at least try to ensure that expensive facilities in one country complement rather than duplicate the capabilities of machines available elsewhere.

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Stanford scandal

SIR—Dr Donald Kennedy (*Nature* **341**, 180; 1989) was not president of Stanford University at the time of the assault on Professor Leonard Hayflick, to which I alluded in a review — he was, I believe, head of the Food and Drug Administration in Washington — but the affair caused an international scandal, and unawareness among faculty members, even those who were not physically present, implies the lack of academic solidarity of which I complained.

I think that if Kennedy reviews the history of this affair, he will find my comments neither unjust nor intemperate.

ALEX COMFORT

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Six of one . . .

SIR—Although one instinctively feels that all information is of value, the data quoted in your recent News and Views comment (*Nature* **341**, 181; 1989) on the British National Survey of Sexual Attitudes and Lifestyles suggest that Mrs Thatcher was right to doubt its value.

You quote the results of a preliminary survey as showing the average (lifetime) number of heterosexual partners as 11.0 for men and 2.9 for women. These figures are literally impossible. A heterosexual union is analogous to a heteronuclear chemical bond, and the total number must be the same if viewed from the male or female end. Thus the average numbers of partners must obey a 'bond consistency' condition

$$c_M N_{MF} = c_F N_{FM}$$

where c is a concentration and N_{MF} the number of female partners per male. Because by empirical observation the concentrations of males and females in the United Kingdom are the same, so must the average number of partners be. The survey results are clearly biased: probably both sexes bias their replies ("lie") in socially acceptable directions. The sociologist you quote was clearly right to be sceptical.

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Mechanical energy from light

In principle, it should be possible to turn radiation directly into mechanical energy, but the best designs so far of machines for doing this consist of paper studies. Somebody should build one.

LIKE mixing chalk and cheese, it grates on expectations of what the natural world is like to be asked to convert light energy directly into mechanical energy, to use sunlight for driving the transmission of a motor-car, for example. Despite present interest in what are called 'renewables', ecospeak for sources of energy alternatives to fossil fuels and uranium, the question is hardly ever asked. And if it were, the answer would almost certainly be a reference to well-tried space technology: make electricity first, and use that to drive the machine.

But that is too cowardly an answer. There is a direct conversion route, even a way of making light drive a piston along a cylinder, as in an internal combustion engine. What can that be? At this point, one must bury the thought that the explanation has to do with the Poynting effect, or with the well-attested truth that photons carry momentum and thus exert a force on whatever absorbs them. Toy radiometers are familiar enough; their aluminium sails do indeed spin round when exposed to light, but few would expect them to do much more — drive a motor-car, for example.

The true design of a light engine proceeds differently, beginning with a working fluid capable of absorbing light and behaving differently as a consequence. Suppose, for example, that one chooses a coloured gas, say a halogen vapour, and exposes that to sunlight. Energy will be absorbed and converted into heat, so that the pressure will increase. In a suitable arrangement, in a cylinder fitted with a piston for example, a modicum of work could be extracted. But the rate at which energy could be extracted from such a device — technically, its power — would necessarily be limited by the speed with which the working fluid could be restored to its condition at the beginning of the cycle, and thus by the rate at which energy is lost by heat transfer to the surrounding medium. Nobody would expect even that to be enough to drive a motor-car.

Is there some way of augmenting that effect? This is the goal at which the designers of the light engine have set their caps. This is how the argument might go (as rehearsed as early as 1973 by A. Nitzan and J. Ross, *J. Chem. Phys.* **59**, 241; 1973). The trick is to choose as a working fluid a chemical species which is an efficient absorber of radiation, but which is itself replenished whenever its effectiveness as

an absorber has been neutralized by having absorbed a photon, so that it is in an excited state.

Specifically, call the absorbing species A and the excited species A*. Suppose that A is itself a dissociation product of another chemical species B, that equilibrium is reasonably well satisfied between the species under the conditions in which the light engine functions and that the reaction by which B is converted into its components (including A) is endothermic. That would mean that, if the temperature increased, there would be more of the product (A*) in the absorbing gas, which would imply positive feedback. One could hope to keep the process of light-absorption going not for milliseconds but for tenths of a second or thereabouts.

That is the point at which three members of the department of chemistry at the University of Chicago get down to business. S. J. Watowich, K. H. Hoffmann and R. S. Berry (*Il Nuovo Cimento* **104B**, 131; 1989) choose as their working fluid the fluorosulphate free radical — stoichiometrically SO_3F , which is nothing but SO_4 in which one O has been replaced by an F. But SO_3F has the interesting property of forming dimers that are neither free radicals nor absorbers of radiation; $\text{S}_2\text{O}_6\text{F}_2$ is simply a reservoir from which absorbing gas can be regenerated whenever there is a tendency for the temperature to increase.

In simple terms, the consequence is chiefly to increase the duty cycle of a light engine based on a light-absorbing molecular gas. But the increase may in principle be very great. If A* is not fluorescent, energy from the excited molecule can escape only by means of molecular vibrational modes, a process of thermal degradation which both restores the original absorbing molecule and which must separately increase the concentration of the absorbing species by shifting the chemical equilibrium.

How to make a light-driven mechanical engine from those ingredients? Watowich *et al.* do their best. Implicitly, they acknowledge that, in the construction of a novel energy-transducer, what matters is not theoretical efficiency but power output per unit of mechanical engineering effort. For that reason, their design study ends up with SO_3F embedded in more than 1,000 times as much buffer gas, perhaps molecular nitrogen (to increase

heat conduction and thus reduce the length of the duty cycle). A large part of the trick is to operate the light engine in a condition in which the working fluid is metastable — endowed with a greater potential than would be found at equilibrium to replenish stocks of SO_3 depleted by the absorption of radiation.

Several difficulties underlie their calculation, at least one of which is philosophical. Ehrenfest's law of detailed balancing implies that, if one can define a molecular species of any kind, one can, in a steady state, equate the total rate at which it is created with that at which it is destroyed. But Ehrenfest was innocent of quantum mechanics, and would not have worried much about the possibility that a dimer in equilibrium with its monomer in the presence of excited versions of the latter might not behave as if the old-fashioned law of mass action were universally applicable. Ordinarily, of course, he would have been on sure ground.

But the mechanical light-engine requires that the conversion of dimer into monomer molecules should be so much more rapid than the rate at which excited monomers lose energy through conversion to intramolecular vibration, and thus enhanced collision frequency, that excited and non-excited monomer states are never confounded. That is probably a mistaken supposition in circumstances in which the effectiveness of the light engine depends on the speed at which the duty cycle can be completed.

These are strictly theoretical objections, of course. They could be answered simply and conclusively by a demonstration that it is possible to build a machine, with some working fluid or other, that will drive a flywheel when it is exposed to a light, perhaps one more intense than sunlight. Sadly, this seems not to have been done. In the tradition of *Il Nuovo Cimento*, Watowich *et al.* hew to the principle that a gram of theory counts for a tonne of experiment, and is usually easier. For what it is worth, the paper efficiency of a working machine turns out to exceed 0.25, just a little greater than that of the best amorphous silicon converters of solar energy into electricity. But it would be more than just fun if somebody were to build one, even a much less efficient version. Then, at least, there would be a tangible incentive to improve on paper performance.

John Maddox

The cell cycle as a *cdc2* cycle

Andrew W. Murray

ORDERLY cell division depends on the coordination of the events of the cell cycle. In eukaryotes this coordination is produced by the regulation of three cell cycle transitions: entry into mitosis, exit from mitosis, and passage through a point in G1 named Start, that commits the cell to initiating DNA synthesis (the S phase). It is now believed that in all eukaryotes each of these transitions is regulated by controlling the activity of a single protein, pp34 — the product of the *cdc2* gene. A model of the cell cycle as a series of closely regulated changes in the activity of pp34 is shown in the figure. On page 39 of this issue¹ Gould and Nurse show that one of these changes is the dephosphorylation of a single tyrosine residue on pp34 and that this reaction facilitates entry into mitosis.

Originally identified as the product of the *cdc2* gene of the fission yeast *Schizosaccharomyces pombe* and the CDC28 gene in the budding yeast *Saccharomyces cerevisiae*, pp34 is now known to be the catalytic subunit of the mitosis-inducing protein kinase known as maturation promoting factor (MPF) or growth-associated H1 kinase (see ref. 2). The other major subunits of MPF are the cyclins³⁻⁷, proteins that accumulate in each interphase and are degraded at the end of each mitosis (reviewed in ref. 8).

Fission yeast requires *cdc2* activity both for passage through Start and for the induction of mitosis. In this organism, entry into mitosis is controlled by the ratio of the activity of two genes: *cdc25*, which stimulates entry into mitosis, and *wee1*, which inhibits it and has homology to known protein kinases⁹. In late interphase pp34 is phosphorylated on both threonine and tyrosine¹⁰. The activation of MPF, both *in vivo* and *in vitro*, is accompanied by dephosphorylation of both amino acids¹¹⁻¹⁴, leading to the idea that the dephosphorylation of pp34 regulates the activation of MPF. Gould and Nurse's new data from fission yeast strongly support this hypothesis.

Their findings can be summarized as follows. In fission yeast whose cell cycle has been arrested in G2 by a temperature-sensitive *cdc25* mutant, pp34 is phosphorylated on tyrosine 15 and on an unidentified threonine. When the cells are returned to the permissive temperature the abundance of both phosphoamino acids declines and the cells enter mitosis. When phenylalanine is substituted for tyrosine 15 of pp34, the mutant protein is no longer a substrate for tyrosine phosphorylation. Strains that carry this mutation grow poorly, even though they divide at a much smaller size than wild-type cells. Moreover, they exhibit a large number of aberrant cell divisions that are presumed to result from premature entry into mitosis. (This phenotype is strongly reminiscent of the 'mitotic catastrophe' induced by the over-expression of *cdc25* in strains carrying mutations in *wee1* (ref. 15).) Finally, the mutant strain can still exit from mitosis, demonstrating that tyrosine phosphorylation of pp34 is not required for this step.

These elegant experiments suggest that the antagonistic effects of *wee1* and *cdc25* may act to control the tyrosine phosphorylation of pp34. This mechanism for controlling entry into mitosis is probably phylogenetically conserved, as homologues of *cdc25* have been found in *Drosophila*¹⁶ and *S. cerevisiae*¹⁷. Unfortunately, the simplest scheme — that the *wee1* product is the pp34 tyrosine kinase and the *cdc25* product is the pp34 tyrosine phosphatase — is unlikely to be true for several reasons. First, the sequence of the *wee1* product resemble serine/threonine kinases rather than tyrosine kinases. Second, the sequence of the *cdc25* product does not resemble known protein phosphatases. Finally, the dramatic effect of overexpressing *cdc25* in strains mutant for *wee1* (ref. 15) shows that *cdc25* must do more than simply reverse the inhibitory effects of *wee1*. It will clearly be of interest to determine whether *wee1* activity is required for the tyrosine phosphorylation of pp34 and the effect of mutating the sites

of threonine phosphorylation on pp34. The ability of fission yeast to divide normally in the absence of both *cdc25* and *wee1* functions⁹ indicates that there are additional controls on the activation of MPF that remain to be discovered.

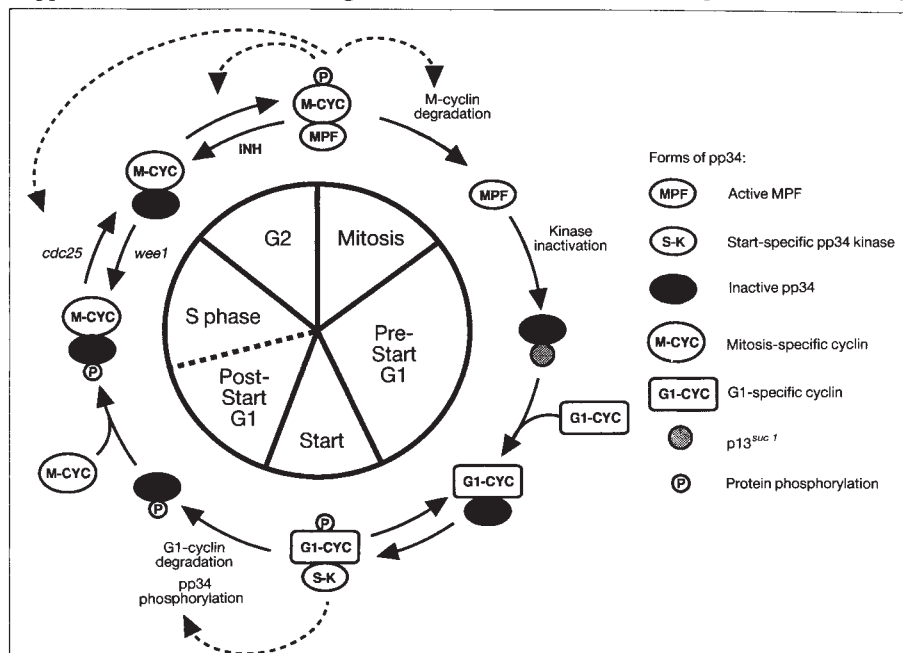
How many biochemical steps are involved in activating the mitosis-specific activity of pp34? Two that we know of are cyclin accumulation^{18,19} and pp34 dephosphorylation^{1,11-14}. In addition, in sea urchins an increase in cyclin phosphorylation parallels the activation of MPF⁷. Thus, in the illustrated model, cyclin (M-CYC) binding to pp34 is required to allow the removal of tyrosine and threonine phosphates from pp34, and the subsequent phosphorylation of cyclin that yields active MPF. The level of phosphorylation of pp34 and cyclin is determined by the balance between kinases and phosphatase activities, so many different regulatory signals can act on this part of the cycle. The *cdc25* and *wee1* genes are shown as stimulating, respectively, the dephosphorylation and phosphorylation of pp34.

In the model, the dephosphorylation of cyclin is mediated by INH, an inhibitor of MPF activation that has been postulated to be a phosphatase²⁰. MPF catalyses the activation of inactive forms of MPF^{1,12,20} and the incorporation of phosphate into the cyclin component of MPF^{4,5,7}, indicating that it may be the physiological cyclin kinase. The model also shows MPF acting indirectly to increase the rate of tyrosine dephosphorylation. These autocatalytic steps would ensure that the rise in the levels of MPF is rapid and not easily

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A model of the cycle of pp34 (outer ring) during the cell cycle. The pp34 molecule lacks kinase activity during most of the cell cycle but becomes active as MPF at mitosis and as a Start-specific kinase at Start as a result of association with phosphorylated cyclins. Note that the active forms can also stimulate their own activation and/or inactivation (---->). The dashed line between post-Start G1 and S phases indicates that no change in pp34 accompanies the onset of replication.

reversible. It is not known whether pp34 dephosphorylation requires cyclin accumulation or pp34 activity, but this question can be addressed experimentally in both intact fission yeast and frog egg extracts.

How is MPF turned off at the end of mitosis? Cyclin degradation is necessary for the exit from mitosis²¹, but the existence of cyclin-free forms of MPF *in vitro*¹², and the transient existence of H1 kinase activity after the destruction of cyclin *in vivo*²², suggest that cyclin degradation is not sufficient for the inactivation of MPF. One candidate member of the inactivation pathway is p13^{suc1}, the product of the fission yeast *suc1* gene. Not only are cells deleted for this gene unable to inactivate MPF²², but the gene product binds pp34, stabilizes the kinase activity of certain temperature-sensitive alleles of *cdc2* (refs 5, 22), and can block the dephosphorylation of pp34 in frog extracts¹⁴. Perhaps, in the absence of cyclin, p13^{suc1} binding to pp34 destroys the catalytically active conformation of the molecule or renders it a substrate for some inactivating activity. In both *Aspergillus* and fission yeast, mutants in type 1 phosphatase genes are defective in the exit from mitosis (see ref. 23). It is not yet known whether the defect in these strains is in the inactivation of MPF or in the removal of phosphate from molecules whose phosphorylation is induced by active MPF.

In mammalian cells and budding yeast, Start is the point in the cell cycle where cells monitor cell size and nutrient availability. What role does pp34 play in this cell-cycle transition? Recent evidence from the fission yeast²⁴⁻²⁶ suggests there are G1-specific cyclins (G1-CYC in the model) that activate a Start-specific

protein kinase activity of pp34 in much the same way as the mitosis-specific cyclins activate MPF. The mechanisms that regulate Start, the biochemical events that are the manifestation of passage through Start, and the substrates of the Start-specific activity of pp34 are mostly unknown. In exponentially growing HeLa cells, the level of phosphotyrosine in pp34 does not increase until the onset of S phase¹¹. This observation raises the possibility that tyrosine phosphorylation of pp34 is a consequence of passage through Start and requires the Start-specific activity of *cdc2*, just as the tyrosine dephosphorylation at mitosis is postulated to require the mitosis-specific activity of *cdc2*. Thus, tyrosine phosphorylation of pp34 would serve as a marker for those

cells that had passed through Start, as well as regulating entry into mitosis.

If complete removal of pp34 phosphotyrosine is dependent on high levels of MPF activity this stigma of passage through Start could not be removed without entry into mitosis. Such a scheme provides a biochemical means for the cell to remember where it is in the cell cycle and thus ensure a regular alternation of Start and mitosis. Finally, if the removal of phosphotyrosine from pp34 is dependent on the completion of DNA synthesis, mitosis could not start until DNA synthesis had finished. □

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ATMOSPHERIC CIRCULATION

Variations without forcing

Marvin A. Geller

ATMOSPHERIC conditions can vary over a vast spectrum of timescales — extending up to the Milankovic cycles operating over millenia and forced by variations in the Earth's orbit. But James and James warn on page 53 of this issue¹ against reading too much into correlations between climate cycles and the periods of plausible forcing mechanisms. In particular, they show that one 11-year circulation cycle that appears to be related to the 11-year solar cycle² can arise naturally from the internal dynamics of the atmosphere.

James and James base their warning on computer simulations of the climate for timescales of 100 years. The physical inputs of the simulation were deliberately simplified to facilitate the long integration times, but included steady solar heating, the effects of friction on the atmospheric circulation, effects due to the Earth's rotation and, in one case, the seasonal cycle. Other variations in external forcing are deliberately excluded. The important difference from previous simulations is the allowance for nonlinear effects in such long integrations.

The resulting atmospheric circulation was examined for periodic variations. This is best illustrated in the spectra in Figs 2-4, on pages 54-55 of this issue, which show the variation of a particular parameter of the circulation — the (0,1) vorticity coefficient which reflects the excess of atmospheric rotation over the Earth's. Despite the lack of any long-period forcing, the circulation varies strongly for periods longer than a year, the strongest response being at around ten years. This is apparent in the simulations that include annual forcing (Figs 2 and 3) and, most significantly, in the simulation in which this was excluded (Fig. 4). This demonstrates, James and James conclude,

that the presence of decadal variations in the atmosphere does not imply that a forcing mechanism of similar period is at work, a conclusion anticipated in long-term simulations of temperature changes from a general circulation model³.

The new results imply that this type of variability can occur as a result of long-lived, non-random effects in the nonlinear atmospheric flow. Unlike linear systems, nonlinear systems can respond to internal and external forcings over periods much longer than those directly being forced. The results of James and James suggest that, although the internal atmospheric instabilities generate internal variability in the fluid flow on timescales of the order of ten days, these are organized by the nonlinear flow into long-lived structures that show variations over timescales as great as ten years: the decadal variations are internally produced.

This result is very relevant to suggestions of a connection between climate and the solar cycle. Two years ago, Labitzke and van Loon² published a provocative paper demonstrating excellent correlation between atmospheric variability at stratospheric levels during the winter months and solar activity. This becomes apparent when the winter data are subdivided into two sets according to the phase of the quasibiennial oscillation (QBO) in the lower-stratospheric tropical wind patterns. The authors have since demonstrated that these strong correlations also exist at tropospheric levels and during other seasons^{4,5}.

There have been several attempts to check the validity of Labitzke and van Loon's statistical approaches. In most cases, their procedures are considered to have passed these tests. In fact, the statistical associations have been sufficiently

- Gould, K.L. & Nurse, P. *Nature* **342**, 39-45 (1989).
- Murray, A.W. *Nature* **335**, 207-208 (1988).
- Pines, J. & Hunter, T. *Cell* **58**, 833-846 (1989).
- Draetta, G. *et al. Cell* **56**, 829-838 (1989).
- Booher, R.N., Alfa, C.E., Hyams, J.S. & Beach, D.H. *Cell* **58**, 485-497 (1989).
- Giordano, A. *et al. Cell* **58**, 981-990 (1989).
- Meijer, L. *et al. EMBO J.* **8**, 2275-2282 (1989).
- Murray, A.W. *Nature* **326**, 542-543 (1987).
- Russell, P. & Nurse, P. *Cell* **49**, 559-567 (1987).
- Draetta, G. *et al. Nature* **336**, 738-744 (1988).
- Moria, A.O., Draetta, G., Beach, D. & Wang, J.Y.J. *Cell* **58**, 193-203 (1989).
- Labbe, J.C. *et al. Cell* **57**, 253-263 (1989).
- Gautier, J., Matsukawa, T., Nurse, P. & Maller, J. *Nature* **339**, 626-629 (1989).
- Dunphy, W.G. & Newport, J.W. *Cell* **58**, 181-191 (1989).
- Russell, P. & Nurse, P. *Cell* **45**, 145-153 (1986).
- Edgar, B.A. & O'Farrell, P.H. *Cell* **57**, 177-187 (1989).
- Russell, P., Moreno, S. & Reed, S.I. *Cell* **57**, 295-303 (1989).
- Murray, A.W. & Kirschner, M.W. *Nature* **339**, 275-280 (1989).
- Minshull, J., Blow, J. & Hunt, T. *Cell* **56**, 947-956 (1989).
- Cyert, M.S. & Kirschner, M.W. *Cell* **53**, 185-195 (1988).
- Murray, A.W., Solomon, M.J. & Kirschner, M.W. *Nature* **339**, 280-286 (1989).
- Moreno, S., Hayles, J. & Nurse, P. *Cell* **58**, 361-372 (1989).
- Cyert, M.S. & Thorne, J. *Cell* **57**, 891-893 (1989).
- Cross, F. *Molec. cell. Biol.* **8**, 4675-4684 (1988).
- Nash, R., Tokiwa, G., Anand, S., Erickson, K. & Futcher, A.B. *EMBO J.* **7**, 4335-4346 (1988).
- Hadwiger, J.A., Wittenberg, C., Richardson, H.E., de Barros-Lopes, M. & Reed, S.I. *Proc. natn. Acad. Sci. U.S.A.* **86**, 6255-6259 (1989).

convincing that some operational forecasts of seasonal mean climate have begun to use the results in making these forecasts⁶. At the same time, strong scepticism has also been expressed about the idea that the correlations imply that solar activity controls the lower atmosphere. The results of the James and James paper provide a good perspective in which to examine this situation.

Van Loon and Labitzke⁴ state the issue very well at the end of their paper:

Some of our colleagues have suggested that what we have detected is a strong tendency for large-amplitude atmospheric variability on a decadal timescale which shows when the observations are filtered through the Quasi-Biennial Oscillation. This is of course a possibility, but such variability has no obvious explanation either. We find it remarkable if an internal oscillation in the atmosphere on a decadal time scale happened to be in phase with the 11-year solar cycle during the very 36 years when we describe it.

James and James clearly do suggest an explanation for atmospheric variability on a decadal timescale: it occurs as a consequence of internal variability. Although it may be improbable that the phase of this internal mode of variability coincides with the phase of solar variability, it is not wildly so. Given that the periods of both variations are about ten years, the probability that they appear to be in phase is about one in five. Furthermore, as both variations tend to be coherent over several cycles, once they are in phase with one another they will tend to stay in phase for several cycles.

The conclusion that Labitzke and van Loon's work implies a large atmospheric response to solar activity forcing has been criticized^{7,8} from two additional perspectives. Baldwin and Dunkerton⁷ have subjected atmospheric data to the same statistical procedure except they correlate them with simulated harmonics of arbitrary period instead of the solar cycle. They find that correlations equal to or even exceeding those found by Labitzke and van Loon between atmospheric variations and solar activity occur in approximately 5 per cent of these cases. Again, the point is that, viewed over a short number of periods, there is a small but reasonable probability of finding phase matching between two unconnected cycles. This is the flaw in concluding that Labitzke and van Loon's correlations are forced by solar activity.

Barnett⁸ shows that there is a significant quasi-biennial variation in tropical sea-surface temperatures, the amplitude of which is modulated over a ten-year period, apparently in phase with the solar cycle. One possibility is that there is a causal relationship between solar variability and the ocean. Or perhaps the decadal atmospheric variability indicated by James and James's results forces the ocean variations. Or the atmosphere and ocean together may display modes of variability that are not present in either individually⁹. Thus, the atmosphere-ocean system has many ways of producing decadal variations internally.

CALCIUM SIGNALLING

Receptor kinships revealed

Donald L. Gill

JUST six years have passed since the action of inositol 1,4,5-trisphosphate (InsP₃) on the release of intracellular Ca²⁺ was first reported¹. Since then InsP₃ has become recognized as a universal 'second messenger' that is released into the cell cytosol following the breakdown of phosphatidylinositol 4,5-bisphosphate in response to cell surface receptor-mediated activation of phospholipase C. InsP₃ is then either further metabolized (see ref. 2) or it interacts with the receptors that mediate intracellular Ca²⁺ release, but whose nature has remained obscure. Recently, Supattapone *et al.*³ isolated an InsP₃-binding protein from the cerebellum that had properties consistent with the receptor. Now an ensemble of independent reports from the laboratories of Mikoshiba⁴, Snyder⁵ (on pages 32 and 87 of this issue) and Südhof⁶, provide important sequence and structural information on this protein and compelling evidence that the same molecule mediates both InsP₃-binding and the release of Ca²⁺ from the endoplasmic reticulum. Surprisingly, the structure of the InsP₃ receptor is also shown to be strikingly similar to the recently cloned ryanodine receptor (RR)⁷, the Ca²⁺ channel of the sarcoplasmic reticulum of skeletal muscle.

An interesting history precedes identification of the InsP₃ receptor; the same protein has been described independently several times from as early as 1976 (see refs 4 and 5). The extraordinary abundance of the protein in cerebellar Purkinje cells provides an explanation for this unusual degree of attention. It is a membrane glycoprotein with a relative molecular mass (*M_r*) of 250,000 (250K) which binds to both heparin and concanavalin A (enabling an effective purification scheme³), and is a major substrate for cyclic AMP-dependent phosphorylation⁸. Various names have been assigned to the protein; Mikoshiba and colleagues

No physical mechanism capable of quantitatively explaining any effect of solar activity on the lower atmosphere has been identified, despite many years of research: this is because solar activity can modulate only weakly the energy put into the atmosphere. Without a plausible mechanism, it is much more likely that we are seeing physically unrelated, though correlated, internal variations in the Sun and internal variations in the atmosphere or ocean-atmosphere system. □

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refer to it as P₄₀₀ (based on a somewhat spurious original size determination), whereas it is also referred to as PCPP-260 (ref. 8) and GP-A (ref. 9). Henceforth, it seems we may now have confidence in referring to it as the functional InsP₃ receptor (InsP₃R).

Furuichi *et al.*⁴ in Mikoshiba's laboratory used three monoclonal antibodies to the purified P₄₀₀ protein to identify complementary DNAs within mouse cerebellum cDNA libraries that expressed the protein. Eventually, the entire cDNA sequence was obtained, revealing an open reading frame encoding a protein of 2,749 amino acids of theoretical *M_r* 313K. From hydropathy profiles, the authors conclude that the protein contains up to nine membrane-spanning domains, but from immunolocalization studies not presented a very large N-terminal region of the protein is on the cytoplasmic face of the endoplasmic reticulum (ER), so that it is only a cluster of up to seven transmembrane domains close to the C terminus that is likely to exist. Localization of the C terminus on the luminal side of the ER was made possible by a nearby epitope and by evidence of glycosylation in the same region. The large cytoplasmic domain probably contains the InsP₃ recognition site (and regulatory elements) with the transmembrane domains providing the Ca²⁺-translocating region; however, assignment of functional domains is still tentative.

Quite independently, Mignery *et al.*⁶ in Südhof's laboratory made several rather intuitive deductions. They noticed that the recently reported¹⁰ partial sequence of a major Purkinje cell transcript named PCD6 (containing an open reading frame encoding 500 amino acids) was for a protein containing several transmembrane domains and perhaps, therefore, an abundant channel protein. With antibodies to the C-terminal peptide, Mignery

1. James, I. N. & James, P. M. *Nature* **341**, 53–55 (1989).
2. Labitzke, K. & van Loon, H. J. *atmos. terr. Phys.* **50**, 197–206 (1988).
3. Hansen, J. & Lebedeff, S. J. *geophys. Res.* **92**, 13345–13372 (1987).
4. van Loon, H. & Labitzke, K. J. *Climate* **1**, 905–920 (1988).
5. Labitzke, K. & van Loon, H. J. *Climate* **2**, 554–565 (1989).
6. Kerr, R. A. *Science* **242**, 1124–1125 (1988).
7. Baldwin, M. P. & Dunkerton, T. J. *Geophys. Res. Lett.* **16**, 863–866 (1989).
8. Barnett, T. P. *Geophys. Res. Lett.* **16**, 803–806 (1989).
9. Schopf, P. S. & Suarez, M. J. *J. Atmos. Sci.* **45**, 549–566 (1988).

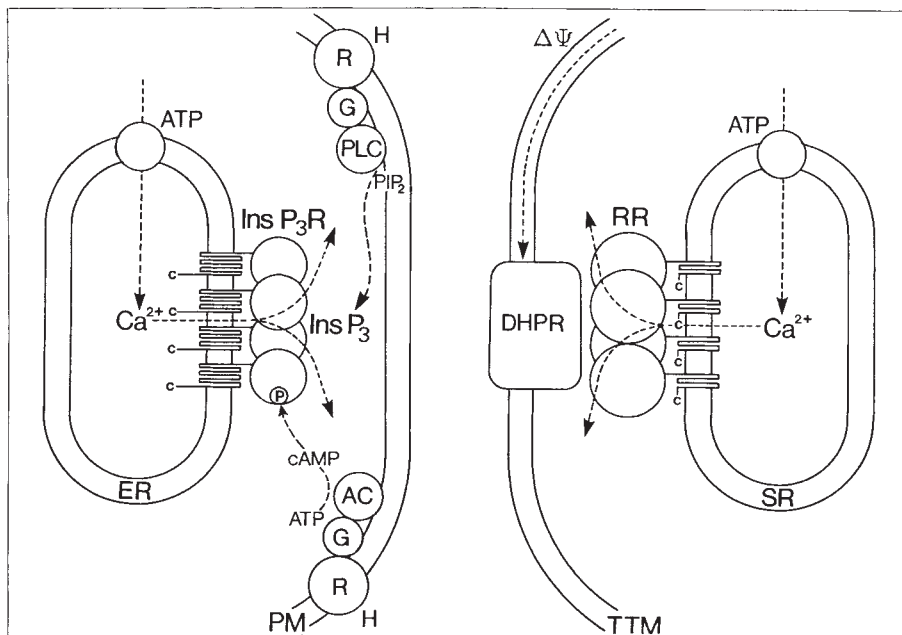


FIG. 1 Comparison of the Ca^{2+} signalling mechanisms through the InsP_3 receptor (InsP_3R) in nonmuscle tissue (left) and the ryanodine receptor (RR) in skeletal muscle (right). In nonmuscle, hormones (H) binding to plasma membrane (PM) receptors (R) coupled to G proteins (G) activate phospholipase C (PLC) and the breakdown of phosphatidylinositol 4,5-bisphosphate (PIP_2) to release inositol 1,4,5-trisphosphate (InsP_3) into the cytosol. The action of InsP_3 on Ca^{2+} release from endoplasmic reticulum (ER) is mediated by the InsP_3R , depicted here as being tetrameric (although of a size suggesting four molecules, the number of subunits in the InsP_3R complex has not been determined). In addition, cAMP, produced by hormonal modification of adenyl cyclase (AC), activates phosphorylation of the InsP_3R and inhibits InsP_3 -induced activation of Ca^{2+} release¹⁹. In skeletal muscle, the initial signal is an electrical depolarization ($\Delta\Psi$) of the T-tubule membrane (TTM), which is detected by the voltage-dependent dihydropyridine-sensitive receptor (DHPR). The signal is believed to be modulated by the RR N terminus (in close contact with the DHPR) into opening of a channel within the core of the RR tetrameric structure^{7,11}. In both systems, the end result is Ca^{2+} release into the cytosol. Note that the C terminus (C) of the InsP_3 is the luminal, whereas that of the RR is believed to be cytoplasmic⁷.

et al. rapidly identified the sequence as part of a heparin-binding protein, of M_r 260K, whose identity with the protein described by Supattapone *et al.*³ was confirmed by its ability to bind InsP_3 . Indeed, its 500-amino-acid sequence differs by only one amino acid from the C-terminal sequence obtained by Furuichi *et al.*⁴.

Is this molecule the functional receptor or merely an InsP_3 -binding constituent of a larger Ca^{2+} -releasing complex containing additional proteins? The answer is convincingly provided by Ferris *et al.*⁵ in the Snyder laboratory, who have now demonstrated that the purified molecule, incorporated into liposomes, mediates Ca^{2+} release in response to InsP_3 concentrations that saturate the binding site. Perhaps most satisfying is the pharmacological specificity: $\text{Ins}(1,3,4)\text{P}_3$, a metabolite of $\text{Ins}(1,4,5)\text{P}_3$ with very little activity *in vivo*, is completely ineffective in inducing Ca^{2+} release in the reconstituted system. Although additional proteins are apparently not required for InsP_3 -induced transport, evidence discussed below suggests that the functional receptor is a multimer of the protein now described.

It comes as a major revelation that the InsP_3R and the RR have similar structures. Over a region of 134 amino acids

(residues 2,541–2,674) in the C-terminal region of the InsP_3R there is 46 per cent identity (64 per cent including conservative changes) with the C-terminal region of the RR. In both cases the region contains the final transmembrane domain. The adjacent transmembrane domains and smaller regions scattered throughout the large N-terminal region of the two molecules are also similar. The total number of possible transmembrane domains in the InsP_3 receptor is in some dispute. As opposed to the seven suggested by Furuichi *et al.*⁴ (which account for oppositely orientated N and C termini), Mignery *et al.*⁶ identify four (the same as the RR), but in either case they all occur within the last 475 C-terminal amino acids. Regardless of the number of transmembrane domains, the overall topology of the InsP_3R , including a large cytoplasmic N-terminal domain and transmembrane domains clustered close to the C terminus, closely resembles the

RR, as shown in Fig. 1. However, the evidence suggests that the InsP_3R C terminus is luminal (see above), whereas the RR C terminus is apparently cytoplasmic. Considering the structural similarity between the C termini and two adjacent membrane-spanning domains, it seems strange that the two regions should be oriented oppositely across the membrane, as it is likely that such regions have important functional correlates (for example, ion translocation).

Another important similarity between the InsP_3R and the RR is that both associate into multimeric structures. There is agreement^{3,4,6} that the M_r of solubilized InsP_3R is around 1,000K, suggesting a tetrameric structure, as shown in Fig. 1. This is particularly reminiscent of the RR, which is believed to function in sarco-plasmic reticulum junctions as a tetramer in an approximately square configuration¹¹, the four subunits possibly surrounding a central Ca^{2+} channel. Indeed, Furuichi *et al.* refer to electron-microscopic evidence for the existence of just such a square-shaped multimeric structure for the InsP_3R . Moreover, a multimeric arrangement is indicated by certain functional studies. Thus, there is good evidence that InsP_3 receptors function cooperatively, occupancy of three or more sites contributing to channel opening¹²; also, the high antagonistic potency of oligomeric heparin (more than 5 nm in length) on InsP_3 effects could reflect a close proximity between groups of InsP_3R molecules¹³. Further assessment of tertiary and quaternary structure of the InsP_3R may reveal much more about the function and control of Ca^{2+} release.

What is the function of the very large N-terminal domain of the InsP_3R ? By analogy (and limited sequence homology) to the N-terminal 'foot' of the RR, which is believed to interact with the dihydropyridine-sensitive receptor in fast muscle (Fig. 1), could this domain be involved in connections to another controlling

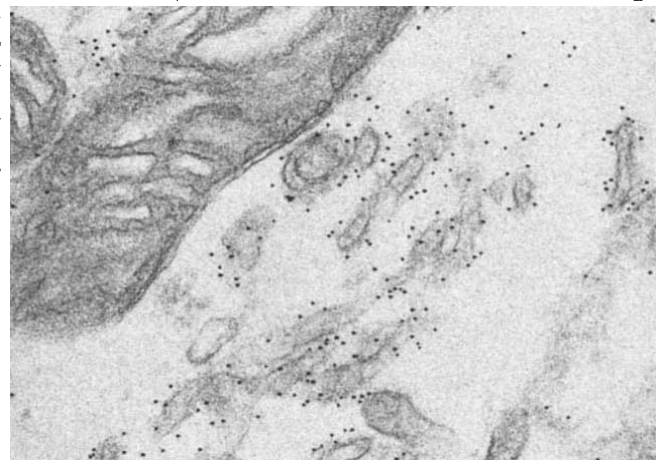


FIG. 2 Localization of the putative InsP_3 receptor in a cerebellar Purkinje cell. Electron micrograph of a cell treated with a gold-labelled antibody to a peptide of the receptor, shows that gold particles are confined to the endoplasmic reticulum. (From ref. 6.)

protein? The lack of any strict organizational configuration of InsP_3 -responsive organelles, compared with the precise organization of sarcoplasmic reticulum feet, would militate against such a role. This raises the question of exactly where within cells InsP_3 receptors are localized. On this point the results are not so consistent. Recent immunocytochemical studies¹⁴, show that the ER is a principal site of InsP_3 R, particularly rough ER (and nuclear envelope), but also peripheral smooth ER (sometimes at locations close to the plasma membrane). The immunolocalization of Mignery *et al.*⁶ is generally consistent with this, although showing more uniform ER labelling (Fig. 2). But Furuichi *et al.*⁴ provide evidence for additional localization in the plasma membrane, a result that supports the functional data of Kuno and Gardner¹⁵ for InsP_3 R-mediated Ca^{2+} translocation across this membrane.

The interesting heterogeneity of InsP_3 R locations does not support the idea of a single specialized InsP_3 -responsive organelle (the calciosome) within cells as suggested by Volpe *et al.*¹⁶. We must also consider the occurrence of complex and dynamic interactions between InsP_3 -sensitive and -insensitive organelles mediated by intracellular G proteins¹⁷; such changes may considerably alter the size and location of InsP_3 -responsive Ca^{2+} pools, and hence the vicinity of InsP_3 receptors.

Lastly, we should consider that much of what has been discussed relates to Purkinje cells, where the massive InsP_3 R density is apparently designed to facilitate extensive granule cell interactions, which

are mediated by glutamate receptors coupled to phosphoinositide breakdown¹⁸ (the cerebellum is thus almost a 'skeletal non-muscle' tissue in terms of specialized Ca^{2+} signalling). Whereas identical InsP_3 receptors appear to exist in most other cell types (S.H. Snyder, personal communication), their organization, control, and distribution may be quite different. □

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1. Streb, H., Irvine, R.F., Berridge, M.J. & Schulz, I. *Nature* **306**, 67–69 (1983).
2. Berridge, M.J. & Irvine, R.F. *Nature* **341**, 197–205 (1989).
3. Supattapone, S., Worley, P.F., Baraban, J.M. & Snyder, S.H. *J. biol. Chem.* **263**, 1530–1534 (1988).
4. Furuichi, T., Yoshikawa, S., Miyawaki, A., Wada, K., Maeda, N. & Mikoshiba, K. *Nature* **342**, 32–38 (1989).
5. Ferris, C.D., Huganir, R.L., Supattapone, S. & Snyder, S.H. *Nature* **342**, 87–89 (1989).
6. Mignery, G.A., Südhof, T.C., Takei, K. & De Camilli, P. *Nature* (in the press).
7. Takeshima, H. *et al.* *Nature* **339**, 439–445 (1989).
8. Walaas, S.I., Nairn, A.C. & Greengard, P.J. *Neurosci.* **6**, 954–961 (1986).
9. Grosz, D.E. & Kelly, P.T. *J. Neurochem.* **42**, 534–546 (1984).
10. Nordquist, D.T., Kozak, C.A. & Orr, H.T. *J. Neurosci.* **8**, 4780–4789 (1989).
11. Lai, F.A., Erickson, H.P., Rousseau, E., Liu, Q.-Y. & Meissner, G. *Nature* **331**, 315–319 (1988).
12. Meyer, T., Holowka, D. & Stryer, L. *Science* **240**, 653–656 (1988).
13. Ghosh, T.K., Eis, P.S., Mullaney, J.M., Ebert, C.L. & Gill, D.L. *J. biol. Chem.* **263**, 11075–11079 (1988).
14. Ross, C.A. *et al.* *Nature* **339**, 468–470 (1989).
15. Kuno, M. & Gardner, P. *Nature* **326**, 301–304 (1987).
16. Volpe, P. *et al.* *Proc. natn. Acad. Sci. U.S.A.* **85**, 1091–1095 (1988).
17. Ghosh, T.K., Mullaney, J.M., Tarazi, F.I. & Gill, D.L. *Nature* **340**, 236–239 (1989).
18. Blackstone, C.D., Supattapone, S. & Snyder, S.H. *Proc. natn. Acad. Sci. U.S.A.* **86**, 4316–4320 (1989).
19. Supattapone, S. *et al.* *Proc. natn. Acad. Sci. U.S.A.* **85**, 8747–8750 (1988).

OCEAN DRILLING PROGRAM

Arc volcanism and rifting

Leg 126 shipboard scientific party*

ACTIVE island-arc volcanoes, noted for their explosive eruptions, characterize the margins of converging oceanic plates. Our knowledge of the nature of volcanism and sedimentation in these areas, and how 'forearc' and 'backarc' basins are formed, has been significantly advanced by recent ocean drilling in the central Izu–Bonin Arc, south of Japan (Fig. 1). Our initial results show that arc rifting is geologically rapid, requiring only two to three million years, and can occur on either or both sides of the island arc. The rifting stage is accompanied by intense volcanism and rapid sedimentation, whereas during subsequent backarc spreading the arc may be relatively quiescent. Volcanism, even explosive eruption, is not restricted to the island arc, but may occur in deep water to either side.

The Izu–Bonin volcanic arc is a product of 50 million years (Myr) of subduction of Pacific lithosphere. Backarc spreading

between 25 and 15 Myr ago in the Shikoku Basin separated the formerly contiguous Kyushu–Palau Ridge from the still active arc (Fig. 1). We drilled in the Sumisu Rift to investigate the geological processes associated with renewed rifting of the arc (before backarc spreading). We also drilled in the forearc basin and discovered that it, too, was formed by rifting, in the mid-Oligocene (28–31 Myr ago).

Four cycles of explosive volcanic activity in the last 250 thousand years deposited glassy and pumiceous silts, sands and gravels which we recovered at the Sumisu Rift sites 790 and 791. This material probably erupted from the nearby Sumisu and/or South Sumisu calderas. The pumiceous unit overlies nanofossil-rich clays, with thin ash beds and scattered pumice and scoria clasts, that date back to 1.1 Myr ago. Sedimentation rates increase exponentially from 80 and 300 m Myr^{-1} at 1 Myr ago to 1,000

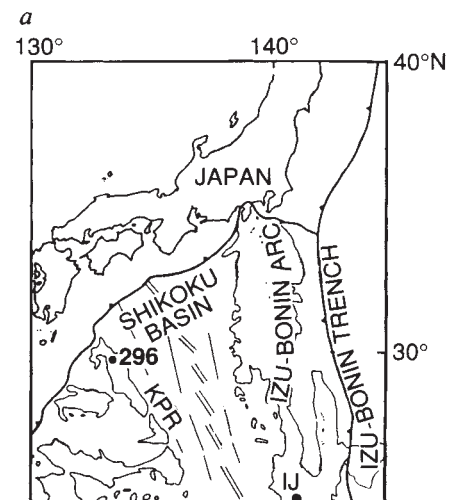
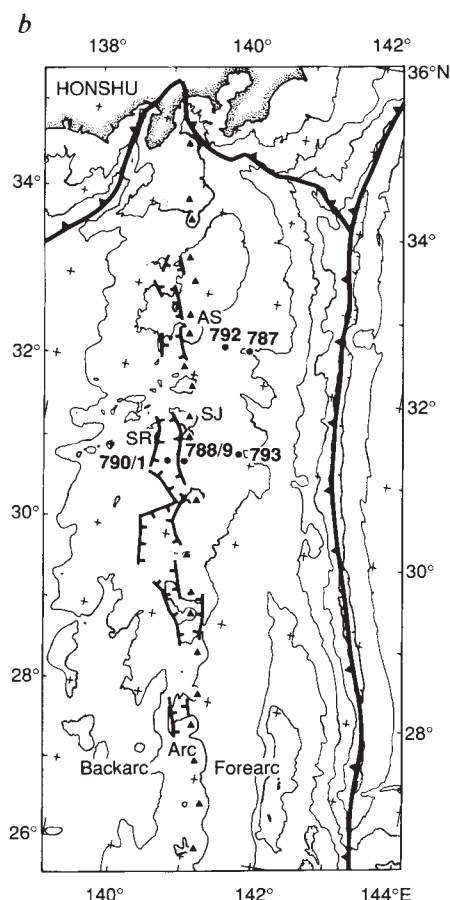


FIG. 1 a, Active plate boundaries and relict spreading centres in the Philippine Sea region. Barbed lines indicate subduction zones and double lines locate relict spreading centres. Basins and ridges are outlined by the 4-km bathymetric contour, except for the Izu–Bonin arc, which is outlined by the 3-km contour. Magnetic anomalies 6 and 6B are shown by single thin lines in Shikoku Basin. U=Iwo Jima; KPR=Kyushu–Palau Ridge; 296=DSDP site 296. b, Bathymetric map showing location of Leg 126 sites. Thick barbed lines locate subduction zones and lines with tic marks indicate rifts. SR=Sumisu Rift; AS=Aoga Shima; SJ=Sumisu Jima.

and 2,250 m Myr^{-1} in the past 100 thousand years at sites 790 and 791, respectively. Benthic foraminifers are consistent with deposition in the present 2-km water depths during the past 730 thousand years and at somewhat deeper levels previously. These data require rapid, differential and accelerating subsidence and sedimentation in the rift half graben. In contrast, only about 30 m of pumiceous gravel have been deposited in the past 275 thousand years, above a major unconformity at the rift margin site 788. The unconformity is the result of margin uplift of between 200–1,700 m (according to benthic foraminiferal data) since the deposition of the underlying pumiceous units 2.35–3.56 Myr ago. The rift formed very quickly, beginning 2 ± 0.5 Myr ago.

A 135-m-thick basalt unit at site 791 probably resulted from submarine fountaining of hydrous lava deeper than 1,500 m below sea level. This unit is composed of clastic basalt froth: both the glassy matrix and clasts are honeycombed with small vesicles, totalling 30–40 per cent porosity. All the tholeiitic basalts at sites 790 and 791 are highly vesicular (20–30 per cent), despite their eruption in deep water, owing to their high pre-eruption water contents. The rift basement basalts may be distinguished from Izu–Bonin arc basalts by their lower barium contents (below 30 parts per million) and higher TiO_2 contents (above 0.95 per cent) relative to MgO , zirconium and vanadium.



The basalts at site 791 have concentrations of barium and rubidium as low as those in mid-ocean ridge basalts and in the most mature backarc basins, indicating that basalts compositions can lose their arc-like geochemical characteristics very early in the rifting stage of backarc basin development. The lowermost rocks at site 791 are arc-derived pyroclastics. Their pumice shards have a eutaxitic texture suggesting hot emplacement, but it is uncertain whether they were emplaced before or during rifting.

Intra-oceanic forearc basins have been avoided by previous drilling legs because of their large sediment thickness. Their stratigraphy, however, records the history of forearc sedimentation, arc volcanism, microstructural deformation and plate motion. To recover this record, and to sample the underlying basement for the first time, we drilled at three sites in the Izu-Bonin forearc (Fig. 2). Studying the basement at the centre of the basin involved drilling the Ocean Drilling Program's deepest hole yet (793B), to 1,682 m below the sea floor.

The three forearc sites documented a characteristic pattern of volcanogenic input: extremely rapid activity before 28 Myr ago; moderate between 28 and 24 Myr ago; minimal between 24 and 13 Myr ago; and moderate and increasingly fast from 13 Myr ago to the present. This pattern is similar to that observed previously at site 296 on the northern Kyushu-

Palau remnant arc ridge (Fig. 1). The early and early-middle Miocene minimum in volcanism corresponds in time with the backarc opening of the Shikoku Basin. This interval is represented in the forearc by nanofossil-rich claystones and nanofossil cherts, quite unlike the surrounding turbidite sequences.

The 200 ash layers (or more) in the upper unit at site 792 are evidence of continuing explosive volcanism since 3 Myr ago. Together with the volcanic record from sites 788-791, they indicate that the Pliocene-to-recent pre- and synrift volcanism and sedimentation along the volcanic front is dominated by rhyolite pumice eruptions. There is no evidence during this time of igneous vents or lava flows between the central Izu-Bonin arc volcanoes, in contrast to arcs such as Japan and the Cascades.

Given the known presence of middle Eocene volcanic highs on both sides of the forearc basin, a big surprise was the lack of Eocene or mid- to lowest-Oligocene (45-34 Myr ago) sediments in the basin. At 715 m below sea floor at site 792, we penetrated an angular unconformity that underlies nearly all the north-central forearc basin sediments. The sediments below this unconformity and down to basement are biostratigraphically and magnetostratigraphically dated as younger than 34 Myr old. At site 793, in the centre of the south-central forearc basin, the sediments overlying the igneous basement are 31 Myr old. Clearly there was no basin before this time. We infer that the surrounding Eocene outer arc and frontal arc highs were formerly contiguous, and that the 40-70-km-wide forearc basin formed when they were separated by rifting in the mid-Oligocene. The stretching event probably affected the whole Eocene basement terrain, from the outer-arc

high to the now-remnant arc ridge (site 296). The newly formed basin, 4-5 km deep according to benthic foraminiferal data, was rapidly (250-300 m Myr⁻¹) filled with turbidite and debris flow deposits, produced by both concurrent volcanism and erosion of the surrounding highs.

The basement at site 793 includes pillowed and massive lavas and more abundant hyaloclastite breccias. Most of the volcanics belong to a high-Mg series of basaltic andesites with boninitic affinities. Others belong to a low-Mg series with tholeiitic affinities. The basement high penetrated at site 792 may form the edge of one of the Eocene-Oligocene frontal arc highs which, like the volcanoes at the present volcanic front, are separated by 20-80 km. The basement lavas at site

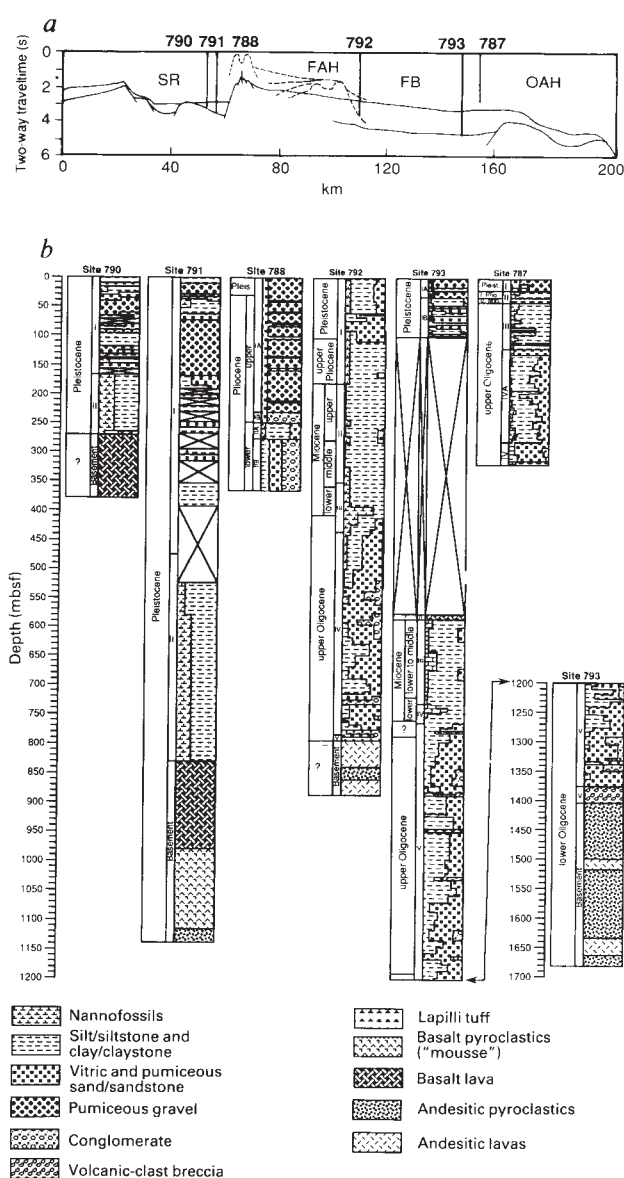


FIG. 2 a, Stylized cross-section across the Izu-Bonin arc-forearc showing location of Leg 126 sites. SR=Sumisu Rift; FAH=forearc high; FB=forearc basin; OAH=outer-arc high. b, Summary lithostratigraphic columns for Leg 126 sites.

792 are massive flows intercalated with hyaloclastite and breccia layers. They are dominantly porphyritic calcalkaline andesites, but are less enriched in low-field-strength elements such as potassium, rubidium, strontium and barium, than most such arc lavas. A middle Miocene or younger basaltic intrusion, serendipitously cored immediately below the casing at site 793, corroborates pre-cruise multichannel seismic evidence for the presence of sills resulting from Neogene forearc volcanism. Izu-Bonin magmas retained a depleted mantle signature throughout the Tertiary.

The pore-water chemistry was closely monitored to detect hydrothermal circulation, especially in the Sumisu Rift. Although indications of circulating sea water were observed in the forearc basin sites 787 and 792, no evidence of hot water circulation was observed at any site. At sites 792 and 793, however, the most extensively altered pore waters yet recovered by the Drilling Programs were extracted from the volcanogenic sediments below 500 and 800 m below sea

floor, respectively. The calcium concentrations are up to 170 and 300 millimolar, respectively. The magnesium concentrations are below detection, sulphate profiles are controlled by the precipitation of gypsum, and at site 793, chloride concentrations exceed 700 millimolar. These highly altered interstitial waters are attributed to the low-temperature alteration of plagioclase and volcanic glass into smectites, zeolites and gypsum, in a virtually closed system, as indicated by the high formation factors. □

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GLOBAL SEA LEVELS

Floodwaters mark sudden rise

M.J. Tooley

GIVEN the wide public concern about the scale of rise in sea level that will occur as a result of the current global warming, many will be intrigued by new studies¹ by J. Shaw of flooding events at the end of the last glacial. From the evidence of erosion and deposition in Saskatchewan, Canada, Shaw concludes that a rise of several metres could have occurred in a few years. Indeed, a catastrophic release of turbulent meltwater from Livingstone Lake, amounting to as much as $8.4 \times 10^4 \text{ km}^3$, could have raised the sea level by 23 cm in as little as a few weeks.

In an attempt to predict the effect of global warming on the sea, earth scientists have analysed the sedimentary record left by past changes. It is clear that the amplitudes of changes vary wildly between different geological eras, although none is as dramatic as that inferred by Shaw.

The maximum rate of rise² of sea level during the recent geological past (the Holocene or last 10,000 years) did not exceed 22 mm a year. However, this mean value obscures the fact that periods of rapid sea-level change are indicated by analyses of empirical data. In my studies^{3,4} in the Ribble estuary, in north-west England, the measured age and altitude of sea-level index points yielded rates of sea-level rise of 34 mm a year about 7,800 years ago; in Morecambe Bay, slightly further north, the rise was 44 mm a year. Corroboration of these rates comes from Limfjord, Denmark, where Petersen⁵

showed the rate of sea-level rise to be 46 mm a year between 8,260 and 7,680 years ago. Finally, for Eastern China, Yang *et al.*⁶ calculate that the rates for the past 20,000 years have peaked three times at 45, 75 and 20 mm a year, between 15,000 and 11,000 years ago.

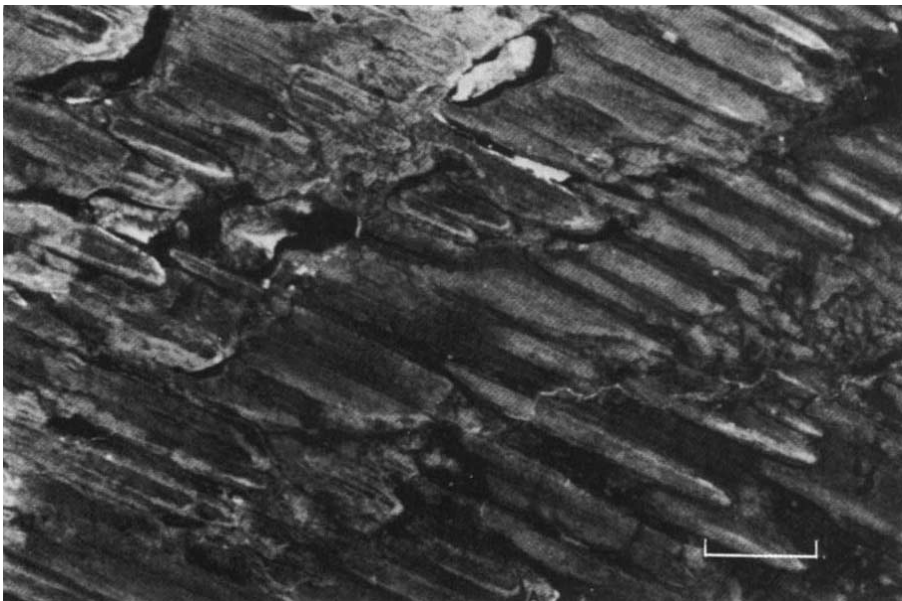
Similar rates are forthcoming for the last interglacial age in Europe. At a recent meeting*, H. Streif (Niedersächsisches

Landesamt Für Bodenforschung, Hannover) concluded that during Eemian II (early in the last interglacial), the sea-level rise was very rapid, possibly 40 mm a year.

These rates of change are several orders of magnitude greater than those calculated for pre-Quaternary periods. For example, during two periods of rapid fall and rise of sea level in the late Cretaceous (over 65 million years ago), rates seem not to have exceeded 9 mm per century⁷.

A severe difficulty in these studies is the time resolution for periods of rapid sea-level change, determined with radiometric and luminescent dating techniques. For my Holocene data from coastal Lancashire, the period of rapid sea-level rise is fixed at one site where radiocarbon assays on organic material immediately subjacent to marine silts yielded ages of $7,820 \pm 60$ and $7,605 \pm 85$ years. Taking the dates with one standard deviation, there is a 66 per cent chance that the rise of sea level occurred during a period of 360–70 radiocarbon years; with 2 standard deviations, there is a 99 per cent chance that this event occurred within 505 years or instantaneously. Also, the altitudes obtained (−9.6 m and −2.3 m) are present-day measurements and have been affected by sediment consolidation, so that the 7.3 m measured sea-level rise is minimal.

Rapid rates of change are not confined to the oceanic part of the Earth's hydrosphere, and those associated with climate require examination. Bryson *et al.*⁸ described the catastrophic disintegration of the Laurentide ice sheet in North America within a couple of centuries about 8,000 years ago, which accounts for the rapid rates of sea-level rise recorded in north-west England and Denmark. The dis-



Aerial photograph of erosional drumlins cut from bedrock in the Northwest Territories, Canada. Shaw uses an analysis of these to infer a massive rate of flooding from the Laurentide icesheet. (Scale bar, 1 km; from ref. 1.)

charge of flood waters, calculated to be $2.0 \times 10^3 \text{ km}^3$, from glacial Lake Missoula is an often-quoted and cyclical event⁹. And Dansgaard *et al.* recently described in *Nature*¹⁰ the abrupt end of the Younger Dryas cold period 10,700 years ago, and concluded that a warming of 7°C in southern Greenland was completed in 50 years and the climate of the North Atlantic region became milder and less stormy in less than 20 years as the sea-ice cover retreated.

Emiliani *et al.*¹¹ showed that the discharge of freshwater into the Gulf of Mexico from the Laurentide ice sheet left a clear signature in the ^{18}O isotope record from the sediment cores. Two peak values in the ^{18}O levels about 15,600 and 13,000 years ago now lead Shaw¹ to suggest that they result from two catastrophic meltwater floods from subglacial landform fields. The evidence for such floods comes from an analysis of extensive fields of drumlins — erosional and depositional features — in northern Canada (see figure).

Shaw concludes that rapid changes of sea level may not be a direct consequence of melting arising from climatic causes, but may arise from glacial hydrological causes alone: that is the accumulation and flow of meltwater in reservoirs beneath the ice. Climatic fluctuations then become, in part, a consequence rather than a cause of meltwater floods which could act as a coldwater lid over the North Atlantic ocean, profoundly affecting climatic parameters, such as temperature and evaporation, the stability of the water column and the oxygen isotope signatures in the sedimentary record.

There are implications for the modelling of future sea-level rise, linked, as it is in present investigations, to predicted global temperature rise. The conservative estimates of future sea-level rise given by Hoffman¹² for AD 2025 and AD 2050 are 13.0 and 23.8 cm, respectively — equivalent to a sea-level rise of about 3 mm a year. At a second recent meeting¹ T. Wigley (Univ. East Anglia) concluded that the best estimate of the sea-level rise by AD 2050 was 24–38 cm, or between 3 and 6 mm a year, which is 3–4 times greater than the so-called global rates during the past 100 years calculated from tide gauge data¹³. The impacts of such rates on natural and embanked coasts of the world cannot be underestimated.

Erratum

It should have been acknowledged that the figure accompanying the News and Views article in *Nature* **341**, 22–23; 1989 by James Scott was based on an unpublished figure of K. A. Hajjar and R. L. Nachman. The published figure contains additional information based on refs 1–3 of the article. In the second paragraph of the article it should have been stated that the apo(a) molecule has 37 or more repeats of plasminogen 'kringle IV' not V1.

These rates approach or exceed the critical rate of 5 mm a year calculated from empirical data¹⁴ from the Lincolnshire Fenland when periods of coastline retreat and marine transgression occurred during the past 6,500 years.

None of these rates, however, approaches the rapid rates of sea-level rise recorded from the stratigraphic record of the last interglacial, the culminating stages of the last glaciation and the early part of this interglacial. The present lack of a precise dating technique leads to the conclusion that even the measured rapid rates of sea-level rise between 22 and 75 mm a year may be conservative. The evidence presented by Shaw tends to confirm this conclusion.

Three questions remain, however.

1. Shaw, J. *Geology* **17**, 853–856 (1989).
2. Kwadijk, J. & de Boois, H. (eds) *European Workshop on Inter-related bioclimatic and land use changes: Final report* (National Inst. Public Health and Environmental Protection, Bilthoven, 1989).
3. Tooley, M.J. *Geogr. J.* **140**, 18–42 (1974).
4. Tooley, M.J. *Sea-level Changes* (Clarendon, Oxford, 1978).
5. Petersen, K.S. *Spec. Publ. int. Ass. Sediment* **5**, 497–503 (1981).
6. Yang, H., Chen, X. & Xie, Z. in *Late Quaternary Sea-level Changes* (eds Qin, Y. & Zhao, S.) 199–212 (China Ocean, Beijing, 1987).
7. Hancock, J.M. & Kauffman, E.G. *J. geol. Soc. Lond.* **136**, 175–186 (1979).
8. Bryson, R.A., Wendland, K.M., Ives, J.D. & Andrews, J.T. *Arctic Alpine Res.* **1**, 1–14 (1969).
9. Baker, V.R. in *The Channeled Scabland. A guide to the geomorphology of the Columbia Basin, Washington* (eds Baker, V.R. & Nummedal, D.) 59–79 (NASA, 1978).

First, Boulton¹⁵ concludes that in the absence of evidence for large-scale drumlin formation associated with the catastrophic release of meltwater from beneath glaciers, drumlins are a product of the interaction between a glacier and sediments on its bed. Shaw's explanation of drumlin formation is one of many. Second, Shaw's hypothesis for subglacial, drumlin-forming floods needs to be extended to Fenno-Scandinavia and tested. Third, is there a potential on the Earth now for catastrophic, subglacial floods raising sea level by 23 cm in a few weeks or several metres in a few years? □

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10. Dansgaard, W., White, J.W.C. & Johnsen, S.J. *Nature* **339**, 532–534 (1989).
11. Emiliani, C., Rooth, C. & Stipp, J.J. *Earth planet. Sci. Lett.* **41**, 159–162 (1978).
12. Hoffman, J.S. in *Greenhouse effect and sea-level rise: a challenge for this generation* (eds Barth, M.C. & Titus, J.G.) 79–103 (van Nostrand Reinhold, New York, 1984).
13. Gornitz, V. & Lebedeff, S. in *Sea-level changes and coastal evolution, Spec. Publ. no. 41* (Soc. Palaeont. Miner., Tulsa, Oklahoma, 1987).
14. Shennan, I. in *The Wash and its environment* (eds Doody, P. & Barnett, B.) 77–90 (Nature Conservancy Council, Peterborough, 1987).
15. Boulton, G.S. in *Drumlin Symposium* (eds Menzies, J. & Rose, J.) 25–80 (A.A. Balkema, Rotterdam, 1987).

* Meeting of INQUA Northwestern Europe Shorelines Subcommittee, Tallinn, Estonia, 18–22 September 1989.

[†] European Programme on Climatic Hazards annual meeting, Cork, Ireland, 3 October 1989.

CONSERVATION BIOLOGY

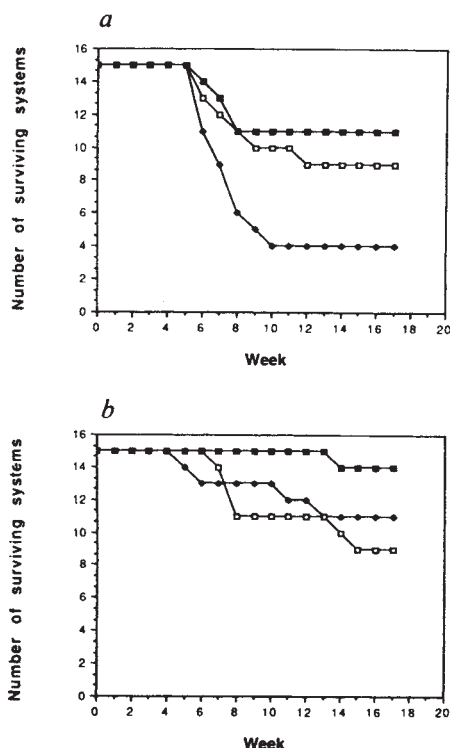
Divided the fruitflies fall

Rolf Anker Ims and Nils C. Stenseth

MAN's destruction of habitat continues to be the leading cause of species extinction^{1,2}. It not only reduces the total area of suitable habitats, but also leads to the fragmentation of large continuous populations into smaller, more or less isolated subpopulations. Dynamics within clusters of such subpopulations can be complex³ because of interactions between numerous factors, such as the degree of subdivision of the subpopulation, the spatial configuration of the habitat fragments and basic population parameters including the rate of increase, dispersal rate, gene flow and social organization. The outcomes of such interactions are very difficult to predict, and such predictions are today almost exclusively derived from simple mathematical models⁴. But Forney and Gilpin⁵, in a recent study of two species of fruitfly, *Drosophila hydei* and *D. pseudoobscura*, have reintroduced the use of experimental model systems, whereby populations are manipulated in simple environments in the laboratory. Their observations have serious implications for the general predictions derived from current methods of analysis of population dynamics.

Mathematical models for predicting population dynamics have been criticized for being too simplistic and inconsistent, often disregarding essential population processes^{4,6}. This criticism is illustrated by the current debate over whether single large reserves or several small reserves are the most effective^{7,8}. Mathematical models present a dilemma: simplistic models have the advantage of being open to thorough analysis but are intrinsically general in nature, whereas complex models that reflect reality more accurately, by way of assumed processes, cannot be analysed thoroughly. The alternative approach, using experimental model systems, strives also to predict population changes from habitat fragmentation by isolating and manipulating the set of factors presumed to be most important. Experimental systems are generally more realistic, and more suited to the study of behavioural responses of organisms to the entirely new conditions created by man. They can, in fact, provide both predictions about real-world phenomena and tests of mathematical models.

In their new study, Forney and Gilpin have compared the fate of *Drosophila* in



The number of surviving replicates in the different experimental systems as a function of time for *a*, *D. pseudoobscura* and *b*, *D. hudei*. ◆, Separated system; □, connected system; ■, large system.

three environments: one in which the original population is divided in two, each subpopulation being confined to a separate culture bottle; a similar system in which the bottles are connected by a corridor that allows migration at the rate of one fly each generation; and a large unified system, in which the initial population and total habitat were twice that of the other systems. Each system, replicated 15 times for both species, was run for 6–8 generations.

Although the total volume of habitat and the total amount of food provided were expected to facilitate subpopulation sizes larger than 20–50 (regarded as the minimum viable size for populations⁶), many of the populations became extinct. Extinctions were, however, less common in the larger system, a twofold increase in population space yielding a sixfold increase in the mean time to extinction. In the separated system large density fluctuations sometimes caused the isolated subpopulations to fall below the minimum viable size, resulting in extinctions due to demographic stochasticity.

Interestingly, the two *Drosophila* species responded differently to the experiment (see figure). *D. hudei* survived better than *D. pseudoobscura* in large and separated systems owing to its higher average population density. *D. pseudoobscura*, on the other hand, survived better in the connected system than in the separated system, and better than *D. hudei* in the connected system,

because of a higher dispersal ability, which tended to dampen the population fluctuations within subpopulations.

Forney and Gilpin conclude, somewhat unsurprisingly, that large, continuous populations are more viable than divided ones. Migration corridors, however, do not seem to compensate for this difference⁹, a finding of particular relevance to the debate over whether such corridors are effective in maintaining populations in conservation areas. Furthermore, the very different response by two closely related species questions the generality of predictions derived from mathematical models.

The robustness of these results can only be assessed when their experimental design has been replicated for other experimental model systems and under different conditions. Future experiments performed under simplified laboratory environments should be complemented by experiments under more complex and realistic, semi-natural field conditions. One such experiment, using an insect predator–prey system, has already been published¹⁰. Our model experiments and those of M. Gaines and R. Holt (University of Kansas) are of the same kind as those by Forney and Gilpin, but use small mammals in manipulated landscapes.

Relative to studies on natural populations, experimental systems are inexpensive in terms of time, labour and money. This facilitates adequate replication and consequently high statistical significance of the results. Of course, experimental model systems can never replace studies on natural systems, but they may aid in the design of large-scale field projects which thereby could more easily focus on the most significant of the ‘jungle’ of processes operating in any natural system. Model systems can also provide the spatial and temporal scales¹¹ required for studies on such natural systems. But, more importantly, experimental model systems may provide an insight of profound value to conservation biologists fighting against the rapidly growing number of species heading for extinction and may indeed be an important complementary approach to the conventional methods of population analysis. □

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1. Red Data Book (The IUCN Conservation Monitoring Centre, Cambridge, 1988).
2. Brown, J. & Roughgarden, J. *Science* (in the press).
3. May, R. *Ecology* **67**, 1115–1126 (1986).
4. Simberloff, D. A. *Rev. Ecol. Syst.* **19**, 473–511 (1988).
5. Forney, K.A. & Gilpin, M.E. *Conserv. Biol.* **3**, 45–51 (1989).
6. Lande, R. *Science* **241**, 1455–1460 (1988).
7. Higgs, A.J. & Usher, M.B. *Nature* **285**, 568–569 (1980).
8. Burke, T.V. *Oikos* **55**, 75–81 (1989).
9. Simberloff, D. & Cox, J. *Conserv. Biol.* **1**, 63–71 (1987).
10. Kareiva, P. *Nature* **326**, 388–390 (1987).
11. Wiens, J.A., Addicott, J.F., Case, T.J. & Diamond, J. in *Community Ecology* (eds Diamond, J. & Case, T.J.) 145–153 (Harper & Row, New York, 1986).

Flash and bang

CONVENTIONAL explosives are dangerously powerful. They have to be, as each small volume of explosive must release enough shock energy to fire the adjacent volume: that is how an explosion propagates. But Daedalus points out that some explosives escape this limitation. A mixture of hydrogen and chlorine, for example, is photosensitive. An intense flash of light can shine through the whole volume and set it all off without any shock having to traverse it. DREADCO's chemists are now exploiting this principle to create new, safe, feeble explosives.

They are synthesizing nitro-substituted polymer-chain molecules with a photosensitive ‘detonator’ group at one end of the chain. When exposed to the right wavelength of light, the detonator photodissociates, and a radical decomposition explosion propagates down the chain. The molecular ‘gain’ (energy released per absorbed photon) can be increased simply by lengthening the chain. A dilute solution of this explosive could never detonate or propagate an explosive shock: its energy density would be too low. But a flash of the right wavelength could fire every photo-explosive molecule in it.

The resulting feeble liquid-phase explosion would heat the solvent to a temperature depending on the initial concentration. Window cleaners, campers and swimming-bath attendants should welcome a harmless powder which dissolves readily in water, and raises it instantly to a chosen temperature when fired by a special flash gun. The sudden expansion would give only a small shock. It should not damage the containing vessel, though the brief molecular agitation might usefully kill suspended bacteria. The disengaged nitrogen and carbon dioxide might also give the water a mild but invigorating fizz.

Dispersed in transparent plastic material, Daedalus's photo-explosive will give a novel photographic film. Exposed in a suitable camera, it will explode only in the bright areas of the image. The sudden local heating and expansion could stamp a metal backing sheet into an instant printing plate. Or it could locally melt the plastic, letting it absorb a backing pigment to create a sort of photographic oil painting.

Daedalus's photo-meltable explosive polymer will also make a wonderful instant glue. The chosen kit of parts need only be clamped together, with strips of plastic photo-explosive in the joints. The DREADCO flash setting gun will beam its light right through the strips in optic fibre fashion. They will melt and expand explosively, impact glueing the joints with molten resin. The whole assembly — table, cupboard or grand piano — will then be firmly bonded in one dramatic flash and bang.

David Jones

The unbegun Big Bang

SIR—While it is most plausible that “the Big Bang is an over-simple view of how the Universe began”, as recently stressed by John Maddox¹, it need not be as “philosophically unacceptable” as he contends. My purpose is to dissent from the common but mistaken idea that Big Bang cosmologies imply “that there was an instant at which time literally began and, so, by extension, an instant before which there was no time”.

In truth, the so-called ‘initial’ instant t_0 corresponds to a singularity of the space-time model (as expressed by the Robertson–Walker metric) which has to be taken at face value: because the model is not defined for $t = t_0$, this value does not belong to its physical time domain. In other words, the continuity, or, more to the point, the contiguity of time breaks down. The range of physical time consists only of the open interval $]t_0, \infty[$; the initial time t_0 is not a moment in the life of the Universe, and does not belong to its past — which *a fortiori* cannot extend before t_0 . As such, this out-of-reach instant may be said to be infinitely remote, irrespective of its finite numerical value on a conventional timescale. This crucial point has been stressed by Misner² who proposes to treat it as “an essential element of cosmological theory” and suggests that we refine our concepts of time accordingly. His plea, however, has been little heard.

This situation is not so new: it is similar to the concepts of limit velocity c , or absolute zero for temperatures T_0 , both of which mark true singularities in the relevant theoretical formalisms and can be considered as infinite, that is unreachable, values. Just as we have become accustomed to the apparent paradox of a zero temperature, below which the concept of temperature breaks down, so we should accept the idea of a time origin before which the concept of time makes no sense.

Furthermore, according to the theoretical framework of modern cosmology, namely general relativity, the choice of coordinates used to describe the Universe is arbitrary. Formally, the spatio-temporal parameters through which the Robertson–Walker metric is expressed, may be modified at will. The usual choice of cosmic time, although convenient, is by no means unique. Thus it is possible, through a suitable redefinition of time, to send back the birth of the Universe to (minus) infinity where, or rather *when* it seems to belong. Such a redefinition, already outlined by Misner², can be derived through a simple group-theoretical analysis designed to restore the additivity of time, a property lost by the conventional cosmic time³. This new, linear time is defined by using the Universe itself as its own clock through its expansion, and leads to a suggestive re-

expression of the Robertson–Walker metric. Naturally, on the linear scale, the lifespan of the Universe is infinite, so that never did the Big Bang begin. Thus, one may agree with the title of the article by John Maddox, *Down with the Big Bang*, provided it is understood as ‘down in time’ (and infinitely so).

Note that no new physics is involved in the introduction and use of linear time rather than cosmic time. Although it might be fruitful for some computational purposes, its main interest is conceptual.

There is certainly a great amount of work to be done for improving or even replacing the standard Big Bang theory as a description of the primitive stages of the

Universe. Let us not confuse, however, the form and the meaning of our theories. If the present ones are certainly open to much scientific questioning, their philosophical status does not seem that disquieting — provided that our own understanding raises to match their subtleties. If, as John Maddox foresees, “creationists will have to retreat to the Big Bang”, we have not too much to fear, as this retreat will be but an endless one.

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1. Maddox, J. *Nature* **340**, 425 (1989).
2. Misner, C.W. *Phys. Rev.* **186**, 1328 (1969).
3. Lévy-Leblond, J.-M. *Am. J. Phys.* (in the press).

Frozen Neanderthals

SIR—Jared Diamond describes in his News and Views article¹, “The recent spectacular discovery of the first reptile to survive natural freezing”. He refers to Storey’s 1988 report² that hatchlings of the Canadian painted turtle in the laboratory can survive the freezing of 52–53 per cent of total body water at -4°C and to comparable findings for certain frogs — facts of great significance to their survival in nature. Diamond concludes that “Although the discoveries of the first freeze-tolerant vertebrates seem to bring the dream of thawing out [and resuscitating a frozen Neanderthal] tantalizingly closer . . . they actually push it even further into the realm of science fiction. Only by a series of specialized adaptations lacking in most animals do the painted turtle and the four frog species confine freezing to the extracellular space and so avoid lethal damage”.

I agree that obtaining viable frozen-thawed Neanderthals is science fiction — not because they lack specialized adaptations, but rather because to survive millennia, they would have to be maintained at temperatures at which biochemical activity ceases, that is, below -120°C . Unfortunately, the very act of cooling a whole animal to such temperatures will increase the solute concentrations in its tissues 30-fold or more over normal and will convert essentially all its body water to ice. The result, at least for vertebrates, will be instant death.

The first report of a vertebrate surviving partial freezing to -4 or -5°C was in 1956, and concerned not a reptile or frog with specialized adaptations but a mammal with no adaptations other than the ability to survive cooling in the absence of freezing to 0°C and slightly below. In that year Smith and Lovelock^{3,4}

reported that 30 per cent of golden hamsters survived, without apparent injury, the conversion to ice of 40–50 per cent of their body water as the result of 40–60 min in liquid baths at -5°C . This result is not dissimilar from those for the painted turtle, except that all four of the latter exhibited no evidence of injury after a 12 h exposure in the partly frozen state to air at -4°C , at which point half the body water was frozen.

Diamond is correct in emphasizing that survival of whole animals (or of most individual cells) after freezing requires that ice be confined to the extracellular space, but this does not demand that the animal possess special adaptations. Rather, it requires: that the first ice forms with little supercooling, and that it first appears in the extracellular space (which is usually the case); that the subsequent cooling rate is low enough to allow sufficient unfrozen cell water to leave the cells osmotically to keep the chemical potential of the residual cell water close to that of the external ice; or that the temperature does not drop to a level at which ice nucleation of the water in supercooled cells can occur⁵. Specialized adaptations do become important in enhancing the ability of cells and organisms to survive greater quantities of extracellular ice (either by the synthesis of cryoprotectants or by plasma-membrane alterations) or by enhancing their ability to supercool.

PETER MAZUR

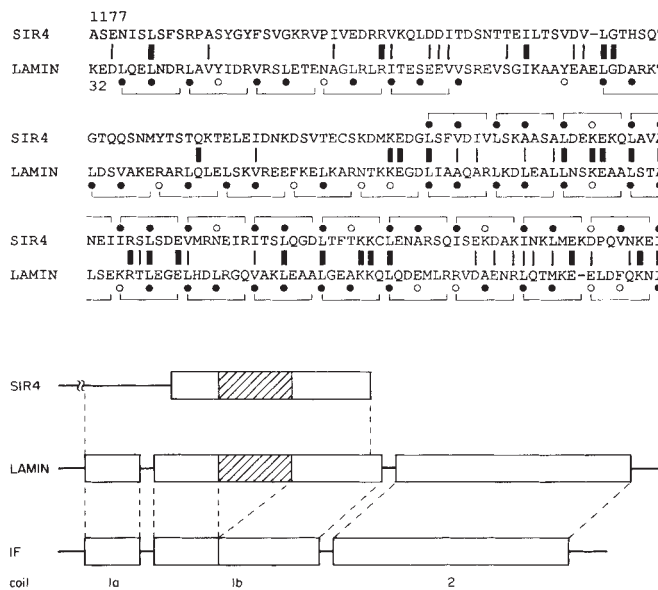
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1. Diamond, J.M. *Nature* **339**, 509–510 (1989).
2. Storey, K.B. et al. *Proc. natn. Acad. Sci. U.S.A.* **85**, 8350–8354 (1988).
3. Smith, A.U. *Proc. R. Soc. B* **145**, 407–426 (1956).
4. Lovelock, J.E. & Smith, A.U. *Proc. R. Soc. B* **145**, 427–442 (1956).
5. Mazur, P. *Am. J. Physiol.* **247**, C125–C142 (1984).

Transcriptional silencing and lamins

SIR—The SIR4 protein in the yeast *Saccharomyces cerevisiae* is required for transcriptional silencing of the *HM* mating-type loci and for high mitotic stability of silencer-containing plasmids. As a result of comparing the sequences of proteins involved in DNA replication and transcriptional control, we have noticed that the C terminus of SIR4 (ref. 1) contains 12 in-phase heptad repeats (brackets in upper figure) within a long region of predicted α -helix (rectangles in lower figure). If the amino acids in the heptad repeat are designated (a-b-c-e-f-g)_n, residues a and d (circles in the figure) are generally hydrophobic aliphatic amino acids — alanine (A), isoleucine (I), leucine (L), methionine (M), valine (V) — and form the basis for coiled-coil interactions. Of the 24

residues in the a and d positions of the SIR4 heptad repeats, 20 are hydrophobic aliphatic amino acids (closed circles in figure) typical of the heptad repeats found in proteins like myosin, tropomyosin and the intermediate filament (IF) proteins. A computer search² revealed that the C terminus of SIR4 is most closely related to the central rod of the human nuclear lamins A and C (23% identity and 36% conservation between residues 123 and 211 of lamins A and C) and that this similarity is not limited to the a and d positions of the heptad repeat. Although lamins A and C are related to other IF proteins³, the greatest similarity between the C terminus of SIR4 and coil 1b of the lamins A and C lies within a 43-amino-acid region found only in the lamins and not in other members of the IF family (hatched region, residues 120–162 of lamins A and C) suggesting that SIR4 is specifically related to the nuclear lamins.



Comparison of SIR4, lamin and IF. Identical amino acids are marked by thick bars, conserved amino acids by thin bars.

Database pollution

SIR—We have noted that a number of entries in the Genbank, EMBL and NBRF nucleic-acid databases contain vector sequences. We compared three vector sequences (plasmid pBR322, phage M13 and λ -phage) with the sequences in the three databases (GenBank release 59.0, EMBL release 18.0, and NBRF-Nucleic release 34.0) and found that overall there are 62 entries that contain unnecessary vector sequence. Most of these entries (52) contain vector sequences which have no serious consequences (that is, vector sequence was left on the end of a submitted sequence). But 10 of the 62 entries contain vector sequences where the authors have made serious mistakes in interpreting their sequence data. These include, a recent entry on GenBank (accession number M19035) that contains a portion of M13, including part of the polycloning site, in the middle of the presumed protein-coding region. Another two (numbers X05793 and X13410) contain vector sequence within supposed coding regions discussed in the papers and six entries (numbers M10384, M14896, M18076, M21517, V00745 and X12660) that

are described as unique sequence are almost entirely vector sequence. These problems would certainly have been avoided if the authors had compared their sequence data with available vector sequences.

In addition to these unwarranted inclusions, there are 9 entries on three databases that contain vector sequences which may be warranted but do not have sufficient documentation.

Inclusion of vector sequence in unique sequence entries adds an unnecessary complication to searching the databases. In some cases it is necessary to include vector sequence in a database entry (such as in fusion constructs), but inclusions of this nature should be clearly annotated. It is not the responsibility of database managers or reviewers to weed out unnecessary sequence information. The responsibility rests firmly with the authors of the sequence.

We have informed GenBank of the sequences in question.

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minus of SIR4 is most closely related to the central rod of the human nuclear lamins A and C (23% identity and 36% conservation between residues 123 and 211 of lamins A and C) and that this similarity is not limited to the a and d positions of the heptad repeat. Although lamins A and C are related to other IF proteins³, the greatest similarity between the C terminus of SIR4 and coil 1b of the lamins A and C lies within a 43-amino-acid region found only in the lamins and not in other members of the IF family (hatched region, residues 120–162 of lamins A and C) suggesting that SIR4 is specifically related to the nuclear lamins.

We note that this region of heptad repeat is an essential functional domain of SIR4 (ref. 1). Furthermore, it is far longer than the 'leucine zipper' found in several transcription factors⁴ and the extent and degree of SIR4–lamin similarity is far greater than that noted by Murre *et al.* for the immunoglobulin enhancer (kE2) binding protein⁵. This region of SIR4 may serve as a dimerization domain, but it is intriguing to consider that it may also directly interact with the nuclear lamina. In support of this, the REP1 protein encoded by the 2- μ M circle plasmid of *Saccharomyces cerevisiae* exhibits a comparable extent and degree of similarity to the lamins and has been shown biochemically to be associated with the karyoskeleton⁶. To explain this, Wu *et al.* postulated that the region of REP1–lamin similarity, found at the C terminus of the REP1 protein, interacts directly with the lamins. It is interesting that the role of REP1 in the mitotic stability of 2- μ M plasmids is strikingly similar to the role of SIR4 in the mitotic stability of transcriptional silencer-containing plasmids⁷. In addition to its effect on mitotic stability, however, SIR4 is required for transcriptional silencing by cooperating with silencer-binding proteins⁸. In light of the similarity between SIR4 and the nuclear lamins, it is not unreasonable to suggest that silencing may involve association of the HM loci with the nuclear lamina through SIR4.

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1. Marshall, M., Mahoney, D., Rose, A., Hicks, J.B. & Broach, J.R. *Molec. cell. Biol.* **7**, 4441–4452 (1987).
2. Pearson, W.R. & Lipman, D.J. *Proc. natn. Acad. Sci. U.S.A.* **85**, 2444–2448 (1988).
3. McKeon, F.D., Kirschner, M.W. & Caput, D. *Nature* **319**, 463–468 (1986).
4. Landschultz, W.H., Johnson, P.F. & McKnight, S.L. *Science* **240**, 1759–1764 (1988).
5. Murre, C., McCaw, P.S. & Baltimore, D. *Cell* **56**, 777–783 (1989).
6. Wu, L.-C., Fisher, P.A. & Broach, J.R. *J. biol. Chem.* **262**, 883–891 (1986).
7. Kimmerly, W.J. & Rine, J. *Molec. cell. Biol.* **7**, 4225–4237 (1987).
8. Kimmerly, W.J., Buchman, A., Kornberg, R. & Rine, J. *EMBO J.* **7**, 2241–2253 (1988).

Bulls, bears and bugs

Julian Davies

Gene Dreams: Wall Street, Academia, and the Rise of Biotechnology. By Robert Teitelman. Basic Books: 1989. Pp.237. \$19.95.

THE rise of modern biotechnology in the 1970s was, with only a few notable exceptions, an American phenomenon. The United States provided a favourable juxtaposition of abundant venture capital and a willingness to take risks, fuelled by the liberal attitude of American universities and their professors. The entire development also rested on the bright young postdocs in molecular biology, who were suddenly provided with a new employment option. Because academic positions were scarce, and conventional industry unattractive, the chance to join like-minded peers in an academic research-style environment under favourable conditions of employment, and without the pressure of grant requests and teaching commitments, was an attractive proposition. The companies themselves were characterized by flexible decision-making, short lines of communication and, above all else, science-based management.

An important difference between the United States and Europe was that in Europe many people sought security and permanence, while in the United States scientists (and investors) were willing to take a chance. The result was that many of them became multimillionaires. For example, the Blech brothers who featured so strongly in the development of the company Genetic Systems (the leitmotiv of Robert Teitelman's *Gene Dreams*) are in the top ten of the list of biotechnology's rich.

In the book, Teitelman describes well the machinations involved in the setting-up, funding and then, in part, the dismantling of a biotechnology company by illustrating the birth, growth (and death?) of Genetic Systems. The company was born with the same scientific idealism as most of the other biotech companies and, like them, with the same naive approach to business and marketing in the real world of commerce. Robert Nowinski, who was one of the founders, provided most of the scientific driving force both in and out of the company. Much of the

Genetic Systems story is built around his ambition and omnipotence, and he is essentially the hero (or scapegoat) of the book.

What I remember best about my years with a biotechnology start-up company (Biogen) was the enthusiasm about the



1,000,000 Units
3,000,000 Shares of Common Stock
and
3,000,000 Class A Common Stock Purchase Warrants

Each unit ("Unit") consists of three shares of common stock, par value \$0.01 per share, (the "Common Stock") and three Class A Common Stock Purchase Warrants (the "Class A Warrants"). The Common Stock and the Class A Warrants will not be separately transferable until September 2, 1981, or such earlier date as may be determined by D. H. Blair & Co., Inc., the Representative of the Underwriters ("Representative"). Each Class A Warrant entitles the holder to purchase at a price of \$3.25, at any time after such Warrant becomes transferable and until June 3, 1983, one share of Common Stock and one Class B Common Stock Purchase Warrant (the "Class B Warrant"). Each Class B Warrant entitles the holder to purchase at a price of \$5.00, at any time from date of issuance until on or before June 3, 1984, one share of Common Stock. The Class A Warrants will be traded in the Over-the-Counter Market on the basis of one Class A Warrant evidencing the right to purchase one share of Common Stock and one Class B Warrant. (See "Description of Securities".)

THESE SECURITIES INVOLVE A HIGH DEGREE OF RISK. SEE "RISK FACTORS."

Prior to this offering, there has been no market for the Common Stock of Genetic Systems Corporation (the "Company"). The Company is in the development stage, it has had no operating revenues to date, and there can be no assurance of future revenues. The initial public offering price per Unit has been arbitrarily determined by negotiations between the Representative and the Company and bears no relationship to the assets, book value, earnings or net worth of the Company or any other recognized criteria of value.

Risk business — the front page of the prospectus released on 4 June 1981 by D.H. Blair & Co., for the flotation of Genetic Systems.

science and the quality of the scientists involved. We hired excellent young PhDs who were willing to change their careers to enter a risky business. There was enormous *esprit de corps*, a biotechnology culture if you will, based partly on the entrepreneurial nature of the business, but also on the fact that we knew that we were involved in tight races with equally talented groups — at Genentech, Cetus and similar companies — to get to a scientific goal and marketable product first. We all knew what the 'products' were. The sense of a race was all the more intense because we were well acquainted with the competition as friends, old academic colleagues or classmates, people with whom we had always had a genial relationship.

Another outstanding feature of the start-up days was the business of financing — the wheeling and dealing to raise private or public funds, the tricks and the enormously hard, and in a sense unsatisfying, task of begging at a high level. We were selling ideas and technology only; it was not until 1983 and 1984 that it became

apparent that market-driven and not technology-driven philosophies would be necessary to convince the analysts and investors that we were interested in making a profit. I recall the endless discussions with the big boys (the Abbotts and Hoechst and Roches) and their apparent conservatism which always heightened our view of ourselves as more innovative, original and better than they were. Even now, scanning the positions vacant columns in *Nature* or *Science*, the ego of young biotechnology companies is obvious. They often begin their advertisements by describing themselves as "leaders", "world-wide leaders", "recognized leaders" or even "recognized world-wide leaders", and they are always "innovative". Modesty is not their strong point. The biotechnology start-ups created an entirely new breed of company, of person and of scientific ideal.

A book which captured all aspects of the exciting and frustrating period, 1975 to 1985, would be an interesting piece of work indeed. *Gene Dreams* is not that book; it is both good and bad. Teitelman is at his best when describing the complex and chicane ways of raising and making money by venture capitalists, and also in his accounts of the internecine relationships

and conflicts between scientific founders and would-be commercial managers of new biotechnology companies. The various phases of the foundation and development of Genetic Systems, and how a company born of such high scientific expectations should have become the source of so much manipulation in the hands of its scientific and business management, are described vividly. Indeed, much of what Teitelman recounts of Genetic Systems provides substance to some well-aided criticisms of many of the biotechnology start-ups. The constant changes of strategies and of organization, for example, are painful memories of mine.

An overriding impression that emerges from *Gene Dreams* is of the simultaneous power and incompetence of biotechnology investment analysts. As Teitelman points out, these highly paid Wall Street soothsayers were no better than fortune tellers. It is at the same time amazing and depressing that a brief utterance from a Wall Street analyst could drive a stock price up or down in a matter of hours and make or lose fortunes. How can such lack

of knowledge yield such power? Who are the gullible, the investment advisors or the investors themselves? Teitelman presents the capriciousness of this business in an excellent manner.

By contrast, much of the scientific commentary in *Gene Dreams* is plain vacuous. Reading parts of the book to colleagues provoked guffaws of laughter. Was it really necessary to depend so much on metaphor and hyperbole? Do oncogenes have "complex splendor"? What is the relationship between a small molecule conjugated to an antibody and a marriage bed? (This sounds like a joke question.) Are some cancer cells like soldiers ducking into a fox hole? Did Genetic Systems really hope to build its business on the "fruits" of early 1980s-style promiscuity? And are viruses the Rosencrantz and Guildenstern of cancer research? In what sense is interferon an endlessly fascinating name like a mythical African Kingdom, or more like Velcro than penicillin (because it is a polymer?). I could continue.

In the book, it is repeatedly stressed that biotechnology has not provided the world with a new industrial revolution (apart from its impact on Wall Street). But an important point is missed with respect to the revolution in basic biology produced by genetic engineering. As a result of this technology, we now have a series of biologically important proteins in pure

form and in unlimited quantity. Many of these proteins were not even known 10–15 years ago, and they have now become part of many significant studies of the human endocrine, immune and neural systems. The list of cytokines, ligands and receptors grows weekly; synthetic genes encoding these compounds are available commercially (one company currently offers 30 or more). The interleukins reported to date number up to seven and we should be in double figures soon. There has been a revolution in fundamental biology as a result of biotechnology — witness, too, our present understanding of

"The concept we started with is now a reality. Our products represent a fundamental shift in medicine, and they're not just working — they're hallmarks."

Reality or hyperbole — Robert Nowinski, founder and provider of much of the scientific driving force behind Genetic Systems, speaks to the shareholders in the 1984 annual report.

the determinants of taste, smell and sight.

Teitelman also insists that the biotech companies have failed when, like Genetic Systems, they sign over their rights (and often more) to large multinationals. Why the crocodile tears? Each of the two parties now has what it did not have before; the one gains financial security, business, organization and marketing services, the other flair, innovation and modern technology. And although he is right in saying that, in business terms, the biotechnology industry has not come up to expectations, that begs the question of who raised the expectations in the first place. The answer, I fear, is mostly the public relations people.

Gene Dreams is undoubtedly a good read. It is relatively free of errors (although I take exception to the suggestion that the word 'biotechnology' was invented on Wall Street) and gives a realistic presentation of what has happened, and is happening, in the venture-capital field with respect to the attempted development of an industry based on biotechnology. Teitelman also provides an accurate picture of the internal and external conflicts affecting biotech companies, and the larger-than-life personalities involved. But this book does not do for its subject what Horace Judson's *Eighth Day of Creation* did for the molecular biology of the 1950s–1970s. For that, someone such as Judson with a more subtle journalistic style, and the ability to capture the scientific excitement as well as the business intrigues, will be needed. There is a cracking story to be told, so why over-embellish and sensationalize it? □

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DNA in flux

J.R.S. Fincham

Mobile DNA. Edited by Douglas E. Berg and Martha M. Howe. *American Society for Microbiology*, 1913 I Street, NW, Washington, DC 20006: 1989. Pp. 972. \$75 (ASM member), \$95 (non-member).

AT THE level of the DNA sequence, chromosome structure is in a state of constant flux, usually slow but sometimes surprisingly rapid. Many processes subversive of conventional genetics have evidently been going on for aeons in the natural world, and over the past several decades descriptions of them have greatly proliferated in the scientific literature. The last attempt to pull the whole complex of phenomena together in a single volume was in 1983, when J. Shapiro edited the monograph *Mobile Genetic Elements* for Academic Press. Now Berg and Howe, supported by the American Society for Microbiologists, have provided us with a more than adequate update.

The 43 chapters by different authors cover several very different kinds of genomic change. First there are the well-controlled DNA rearrangements through which organisms of various kinds can create adaptive diversity from a common initial cell type. These include, in vertebrates, the differentiation of cells of the immune system (only immunoglobulin heavy-chain-class switching is dealt with here) and many examples of DNA-based clonal diversification in bacteria and protozoa.

The second kind of DNA mobility is due to the activities of parasites, and includes the integration and excision of the genomes of temperate bacteriophages such as phage- λ and Mu-1, the insertion of reverse transcripts of retrovirus genomes into the chromosomes of animals and plants, and the so far isolated case of insertion of a plasmid DNA segment into plant chromosomes as part of the infection strategy of *Agrobacterium tumefaciens*. From the point of view of the parasite, if not of the host, these processes are precisely controlled and clearly adaptive.

We enter a much more problematical area when we come to the great range of repetitive DNA sequences, present to a greater or lesser extent in virtually all organisms, that have been described, always in derogatory terms, as 'selfish', 'ignorant' or 'junk'. What they all have in common is the capacity to appear at new sites in the genome, though the frequency with which they move varies enormously. Some of them look like dead or crippled retrovirus genomes or, like the retrovirus-related elements best studied in yeast and *Drosophila*, seem to have settled to the role of benign molecular parasites, no

THE DICTIONARY OF CELL BIOLOGY

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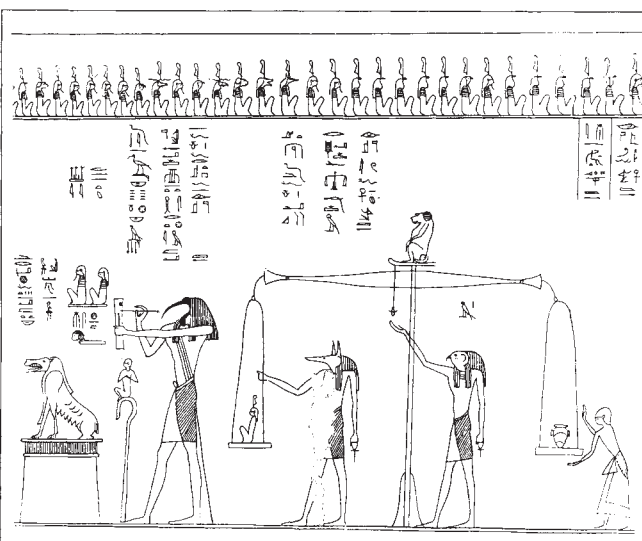
J.M. Lackie and J.A.T. Dow

December 1989, c.320 pages,
Paper: ISBN: 0.12.432561.0, £9.95
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The Dictionary of Cell Biology presents nearly 4000 terms which cover classical cell biology and spill over into the adjacent fields of molecular biology, genetics, neurobiology, physiology, immunology and pathology. Synonyms are provided where necessary and cross-references link terms that are related. Essential for students and researchers in cell biology and related areas.

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Academic Press, 24-28 Oval Road, London,
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Matters of the heart — Ancient Egyptians believed the heart to be the seat of thought, emotion and intelligence — essential to both the living and dead. This reproduction of a twelfth-century BC papyrus shows a heart from the dead being weighed against a feather which represented law, truth and order. Entry to the afterlife was allowed only if the feather outweighed the heart. Taken from *The Medical Skills of Ancient Egypt* by J. Worth Estes, published by Watson.

longer forming infective particles and only occasionally moving to new chromosomal sites through transcription into RNA and reverse-transcription back to DNA. Others, like the IS elements and transposons of bacteria, the P element of *Drosophila* and the numerous transposable elements now identified in maize and antirrhinum, seem to move quite actively by excision and reinsertion without an RNA intermediate. They resemble the retrovirus-related elements only in encoding the enzymic apparatus for their own movement. Whether the so-called LINES (long interspersed sequences), so abundant in mice and men, are self-moving remains a moot point — they have long open-reading-frames that could code for something with a rather remote resemblance to a reverse transcriptase.

Beyond these there is a host of apparently 'ignorant' sequences which are strongly suspected of having been amplified and dispersed willy-nilly by virtue of reverse transcriptase activity provided by something outside themselves. These elements include pseudogenes (excluded from this volume, presumably because they have no ability to move again) and a great host of SINES (short interspersed sequences) that evidently derive from abundant small RNA molecules transcribed by RNA polymerase 3. SINES are thought to be capable, at least until they decay by mutation, of multiple rounds of proliferation because they carry their own transcriptional promoters with them when they move.

Berg and Howe's compilation extends somewhat beyond these topics to include chapters on illegitimate recombination of DNA in prokaryotes and eukaryotes. M. Meuth's article on eukaryotes is particularly interesting but, surprisingly, does not address the question of how cells of higher eukaryotes are apparently able to integrate into their chromosomes any random bit of non-homologous DNA that is fed to them. Perhaps there is nothing yet to be said on this strange property, on

which nearly all current transgenesis experiments depend.

Given that the net was cast so wide, it is perhaps a pity that certain other topics peripheral to the main theme were not included. For example, the tandemly repetitive species-specific 'satellite' DNA arrays found particularly in heterochromatin must certainly have resulted from amplification and dispersal relatively recently on an evolutionary time scale. The mechanisms through which this may have happened are at least worth thinking about. There could be a connection with the spectacular specific gene or chromosome-segment amplification that mammalian cells are capable of under special conditions of selection or stress. Such amplification arguably deserved more than the brief mention and diagram that it gets in Meuth's article.

All of the chapters are by authors with deep experience of their respective subjects. Some, for example Varmus and Brown's 56-page article on retroviruses, with its 397 references, might have been marketable as admirably concise mini-monographs in their own right. The general layout of the book is attractive and clear, much in the style of *Microbiological Reviews*, with a detailed contents page preceding each chapter and plenty of headings and subheadings to help one find particular bits of information.

My only serious reservation about this authoritative and useful volume is that no attempt has been made, either implicitly in the arrangements of the chapters, or explicitly in the form of a general introduction or summary, to provide a scheme of classification of the very diverse phenomena described. Taxonomy may or may not represent real phylogenetic or mechanistic relationships, but at least it helps one to get a mental grip on what may otherwise seem a maze of phenomena. □

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Between Tethys and Pacific

A.M.C. Şengör

Geological Evolution of South-East Asia. By Charles S. Hutchison. Clarendon: 1989. Pp. 368. £65, \$135.

Geological Evolution of South-East Asia forms a connecting link between the two previous volumes in the *Oxford Monographs on Geology and Geophysics* (*The Geology of China*, published in 1986, and *Phanerozoic Earth History of Australia*, 1984). It is a most welcome addition to the series, for it deals with an area about which no modern comprehensive regional treatise exists.

Hutchison's text consists of nine principal chapters arrayed in an unusual order. Following a seven-page introduction, which is devoted mainly to a review of the history of geological studies in the individual countries covered in the book, Hutchison goes on to describe the familiar late Mesozoic and Cenozoic tectonic features such as island arcs, back-arc basins, small oceans, microcontinents, and the main fracture systems of South-East Asia. Chapter 3 is reserved for a description of the Cenozoic sedimentary basins, and here I applaud the author's view that "it is preferable to use terminologies related to the elements of plate tectonics". He erects 12 categories of basin, representing divergent, convergent and intra-plate environments. A curious omission here is strike-slip related basins and some of the more prominent of such basins in South-East Asia are hidden under Hutchison's only unfortunate category, that derived from an influential but misleading basin classification, the uninformative "Pannonian-type".

Chapter 4, "Phanerozoic Tectonic Framework", is an introduction to and a synopsis of the following five chapters. Hutchison defines the sutures and the suture-bound blocks in the region and gives a review of the tectonic evolution, much of which seems to be based on Y.G. Gatinskiy's doctoral thesis published in Russian in 1986 and to have inherited its schematism. Although Hutchison pays lip service to terranology ("the region represents an assemblage of terrains"), he wisely avoids the much-ventilated 'terrane analysis' and sticks to describing the suture-bound blocks of both Cathaysia and Gondwana-Land affinities, plus the intervening sutures together with their ophiolites. Finally, Chapters 8 and 9 cover "The Great Sunda-Pacific Volcanic Arcs", and Hutchison's long-term love, "Granite and Associated Plutonic Rocks".

I have mostly good things to say about this book. In the preface, the author

candidly states that

This is not a standard regional geology, and the selection of the title allowed me to emphasize tectonic models, but at the same time to document the stratigraphy and igneous events. I have always taught my students that every aspect of geology involves interpretation . . . This book is, therefore, a personal interpretation of the region.

In addition, it contains much information on Tertiary oil and gas basins, but "Unfortunately, mineral deposits [Hutchison's speciality] have been excluded due to length constraints". Hutchison is meticulous in his documentation and conscientious in his presentation. I disagree with a number of his interpretations, but am happy to see that he almost always mentions the alternatives and gives references to them.

The 30-page reference list at the end of the book is an invaluable introduction to the scattered sources of information in South-East Asia, and some of the few critical omissions (for example, the 1986

thesis of N. Sikumbang on late Cretaceous obduction in the Meratus Mountains) are readily excusable given the author's remoteness from large geological libraries. The volume is well produced, and is richly illustrated by line drawings of maps, stratigraphic columns and sections; 17 photographic plates are also included. A drawback here, though, is the paucity of seismic reflection profiles.

All those interested in the geology of South-East Asia, especially its terrestrial parts, or in Tethyan tectonics in general, will benefit from reading Hutchison's book. Because of the tremendous tectonic diversity it documents, it will also be a handy reader for a general course on tectonics at the post-graduate level. □

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Growing pains

John de la Mothe

E. W. R. Steacie and Science in Canada.

By M. Christine King. *University of Toronto Press: 1989. Pp. 243. \$40, £24.*

In 1939 when the photochemist, E. W. R. Steacie, left McGill University to become Director of the National Research Council's (NRC) Chemistry Division, science in Canada was just beginning to become a force to be reckoned with. Until the late 1930s, Canadian science had been in an embryonic state and was far from being world-class. Christine King's intention is to tell how, in the next 20 years, a transformation came about in large part because of Steacie's efforts.

The shallowness of science's roots in Canada can be gauged from the situation in 1918. Then, only 11 doctoral degrees in science had been granted in the entire country; only two or three universities had what could be considered extensive research programmes; and the total value of industrial research and development performed in the country was estimated at less than \$135,000 per year. The NRC — which had been founded in 1916 and which had been given the broad mandate of stimulating and coordinating research and development in Canada — had yet to make much of a mark. But what had begun as a one-person operation with an annual budget of \$91,600 had grown, by the time of the outbreak of the Second World War, to an institution with a staff of 300, a budget of nearly \$1 million and a substantial building of its own. The NRC was to gain further strength during the war

and afterwards burgeon into a centre of excellence to which researchers from five continents would gravitate.

The first years of peace were good for Canada. The economy was booming, money was plentiful and the ministers of the Liberal government were sympathetic — even enthusiastic — about building the NRC. The research assistance which the NRC had begun to give to Canadian industry during the war continued with increasingly recognizable (and recognized) effect. Agricultural machinery, mining and transport equipment, construction standards, and pulp and paper processing were all areas which became acknowledged Canadian strengths worldwide.

During this period, industrial research was only one facet of the NRC's activities. With the energetic involvement of Steacie as Director, and later as Vice-President and then (in 1952) as President, the NRC actively promoted the development of a university research system in Canada. Among the obstacles were the antagonism of the big universities to the NRC, as well as the growing sentiment among politicians that the NRC's large budgets should first be questioned, and then, if approved, fed directly into Canada's economic development (and not its ivory towers). In the early 1950s, Steacie negotiated in masterly fashion with the universities and with government officials on three basic propositions: that the universities' duty is to "give society what it needs, rather than what it wants"; that the university is the cornerstone of any nation's research system and capability; and that Canada would suffer a severe shortage of scientific researchers, and run the risk of losing ground economically to other

nations, unless something was done.

Steacie's arguments were hard fought but ultimately well received. Of particular importance was his idea of the NRC's postdoctoral fellowships, which would bring scientists to Canada on "princely stipends". As a result of this programme and the NRC's growing research reputation, many researchers not only came to Canada, but stayed, thereby helping to build the university system.

All was not plain sailing, however, either for the NRC or for Steacie. In 1958, the incoming Conservative government immediately began moves to bring the NRC into the civil service. As in Britain, the scientific civil service was a poor relation in terms of both salary and stature. It was a change of policy that was bitterly resisted as it spelt greater management and political accountability for NRC's researchers, and the end of their freedom of scientific enquiry.

In the end, the political pressures won out. But in 1962, when Steacie died of cancer, he had undoubtedly become the greatest figure in Canadian science. In his own modest terms, he had made the NRC one of the main engines of the Canadian research system, and so, as Christine King says, helped "Canada come part of the way along the road from the ox-cart to the bulldozer".

Christine King, who was tragically killed soon after completing the manuscript of this book, attempts to tell the story of a now-powerful institution and of the men who guided it. Above all, her intention is to give some insight into the powerful personality of the NRC's leader at a time when science in Canada was in its formative years. Sad to say she succeeds only in part. Although the book is thoroughly researched and reads well, it fails to convey either a rounded picture of Steacie's character or the actual tensions between science and government that affected his ambitions and directed his choices (and that still exist in Canada today). To some extent, this is the consequence of the style of the book. Scientific biographies such as this tend to portray the protagonist in an heroic light, blurring the background of other personalities and the politics which are an essential part of the overall picture. Nonetheless Dr King's contribution is to recall an important individual at an important time in Canadian history, and for that her book is to be welcomed. □

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• Volume 1 in the series *Aperiodicity and Order* was reviewed in *Nature* earlier this year (338, 550; 1989). Academic Press has now published the second volume, *Introduction to the Mathematics of Quasicrystals*, which, like the first, is edited by Marko V. Jarić. Price is \$59.50, £42.50.

An analysis of geophysical experiments to test Newton's law of gravity

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Signals reported as evidence for a non-newtonian 'fifth' force at a North Carolina television tower and elsewhere can be explained in a conventional way by postulating small density variations underground. The assumptions employed in earlier analyses which pointed to a failure of the inverse square law are examined and found to be difficult to justify.

THREE recent geophysical experiments to test Newton's law of gravity rely on gravity measurements made along a vertical path: in a 1,000-m deep mineshaft at the Hilton mine, Queensland, Australia¹⁻³; on a 600-m television mast, the WTVD tower near Raleigh, North Carolina, USA^{4,5}; and in a 2,000-m borehole into the Greenland ice cap, at the Dye-3 radar station⁶. According to classical potential theory, the values obtained on the vertical profile should be exactly predictable from other gravity observations made in the vicinity and, in the case of the underground experiments, knowledge of the density around the shaft. But it has been consistently asserted that prediction and measurement fail to coincide, and this has been taken as strong evidence for the necessity of a small modification to the inverse square law, along the lines called for by a quantum theory of gravity⁷ or by introducing a totally new force of nature⁸. The predictions, however, depend on some assumed model of the gravity to interpolate between the survey points. The best calculations so far⁵ draw on an unverifiable statistical model. We offer here an alternative test based on the range of permitted densities encountered in the Earth. We apply the test to the television tower experiment, and we show that conventional physics can account for the observations. The same conclusion holds for the Greenland experiment⁶. We are unable to carry out a comparable test for the Australian experiment^{2,3} because the original survey data are proprietary; in any case, correction in a mine experiment would require downward continuation, which is intrinsically unstable. We conclude that the present collection of geophysical experiments provides no compelling evidence for discrepancies in the inverse square law of gravity.

Predicting gravity on a vertical profile

The value of the acceleration due to gravity, g , near the Earth's surface is governed by the Earth's rotation and its mass distribution. Traditionally geophysicists correct gravity observations for the Earth's rotation and the attraction of a model Earth; what remains is the sum of the attractions from nearby topography, from density variations in the underlying rock and, for mine and borehole observations, from the surrounding rock. In the case of underground measurements, when the density of the local material is accurately known, its attraction can also be removed. Having been corrected in this way, the gravity field obeys Laplace's equation. According to potential theory⁹, the field away from the Earth's surface can then be predicted perfectly if the field is known everywhere on the surface, either by upward continuation for values above the surface, or analytic continuation for those below it.

In practice, exact prediction cannot be made with potential theory: in addition to the inevitable noise in the measurements, one must contend with the need to evaluate an integral that

demands knowledge of the field at every point on the surface, not just at the observation sites; different modes of interpolation must lead to different results. Because the prediction from the surface field is inexact, one cannot decide whether or not it is in agreement with the observed value (and so test Newton's law) unless it is accompanied by an estimate of accuracy; this need is particularly acute in the case of downward continuation, which is a well-known example of an ill-posed problem^{10,11}. An uncertainty estimate requires a characterization of the gravity field between the measurement sites; without it any gravity value on the vertical path is compatible with every finite surface survey. To see this, imagine expanding the gravity field in a basis of harmonic functions, such as the Fourier-Bessel set used by Romaidès *et al.*⁵ in their second method of prediction. If enough basis functions are included, one can always find a model field simultaneously in perfect accord with the surface data and the vertical-profile data by incorporating the tower values into the data set and fitting them rather than predicting them. Incidentally, this example illustrates why the prediction of the Fourier-Bessel expansion cannot be equipped with a satisfactory uncertainty estimate: any anomaly can be matched perfectly. Such a procedure, however, runs the risk of generating fields with wild oscillations away from the sample points, but as we shall show, such bad behaviour is not a necessary feature of models satisfying both tower and surface observations in this case.

The first method of analysis of Romaidès *et al.*⁵ is an example of upward continuation, in which the introduction of an explicit-field model enables the authors to compute uncertainties. They use least-squares collocation (LSC) to predict the values of g along the tower. In LSC, the gravitational field is taken to be the realization of a homogeneous, isotropic statistical process¹². The estimated power spectrum of the process provides the necessary information about the behaviour of the gravity field between the survey sites. Thus, the predictions of LSC depend not only on the correctness of Newton's law but also on the validity of an elaborate statistical model. As the predictions fail to match the observations, logic dictates that one (or both) of these assumptions must be wrong. To claim that the failure impugns the inverse square law is to regard the statistical model as unavailable. Yet one cannot verify objectively the applicability of a statistical theory of stochastic processes to an object like the Earth's gravity field, which has only one realization. Moreover, it is self-evident that the required spatial homogeneity fails drastically on scales longer than 100 km, as the site is on the boundary of two quite different geological provinces, a major batholith to the north-west and coastal sedimentary sequences to the south-east. Furthermore, as we shall show, there are reasonable (but presumably nonstationary) classical surface fields consistent with the g -values on the tower. We therefore believe that there is no compelling reason to place greater confidence in the statistical model than in the inverse square law.

The second method given in ref. 5 for upward continuation, expansion in a Fourier-Bessel series, cannot be regarded as providing reliable confirmation of their LSC results because, as we mentioned, the uncertainty of its results is uncontrolled.

An alternative test

We propose an alternative strategy, based on the investigation of the field sources—that is, the density distribution in the

ground—rather than on a statistical model of the gravity field. This is an indirect way of constraining the interpolated field. An apparent difficulty with this idea is the severe non-uniqueness of the gravimetric inverse problem: the interior density structure cannot be obtained even from complete knowledge of g above the ground. However, geology and geophysics can provide a trustworthy upper bound on the density contrast to be expected in the Earth. We therefore seek a mass distribution whose maximum density is as small as possible; if this solution exhibits a density higher than the largest geologically plausible one, we may question the validity of conventional physics. All earlier tests developed for this problem use the survey data to predict values on the vertical profile; by contrast, we propose to find a single model consistent with the complete gravity data set based on classical theory and to examine that model for implausible consequences.

The basis for a quantitative theory has been given in the method of ideal bodies^{13,14}. Here we extend the original approach to handle observational uncertainty. The fitting of the entire set of observations to a density model is accomplished by solving the convex optimization problem¹⁵

$$\min_{0 \leq \Delta\rho \leq \Delta\rho_0} \sum_{j=1}^N [\Delta g_j - F_j[\Delta\rho]]^2 / \sigma_j^2$$

where

$$F_j[\Delta\rho] = \int_{D \in \mathbb{R}^3} \frac{G \hat{n} \cdot (\mathbf{r}_j - \mathbf{s})}{|\mathbf{r}_j - \mathbf{s}|^3} \Delta\rho(\mathbf{s}) d^3\mathbf{s}$$

The function to be minimized measures the standardized discrepancy between the observations and the predictions of the model: Δg_j are the corrected gravity observations; F_j is Green's function relating the interior density contrast $\Delta\rho$ to the gravitational field; σ_j is the assigned uncertainty of the j th measure-

ment, which is at position $\mathbf{r}_j$. The minimization is carried out over all density contrast functions $\Delta\rho(\mathbf{s})$ bounded above by the constant value $\Delta\rho_0$. Practical computations are performed in an approximate way by dividing the region D underground into a finite set of cuboidal cells within which the density is constant; thus we approximate the original problem with a finite-dimensional quadratic program¹⁶. The parameter $\Delta\rho_0$ is varied until a satisfactory fit in the sense of χ^2 is attained; it can be shown¹⁷ that this parameter corresponds to the smallest maximum density consistent with the measurements at the achieved level of misfit. Negative density contrasts can be handled in the obvious way.

Application to the underground experiments

The first application of the new test was reported by Ander *et al.*⁶, in a discussion of the Greenland experiment. The corrections to the gravity observations discussed above were performed, including the terrain correction applied to account for the gradients caused by the irregular shape of the ice/bedrock interface; the preliminary interpretation of the remaining gravity gradient of $\sim 2.5 \text{ mGal km}^{-1}$ was that a non-Newtonian effect with the opposite sign to that found in the Australian study was the cause. However, subsequent examination of the corrected surface gravity observations revealed a compact positive gravity anomaly to the north-east of the drill site. Ideal-body calculations showed that the presence of this anomaly required density variations in the basement rock that had to be as large as 0.3 g cm^{-3} ; it was shown that a density contrast of this amount in the bedrock could also account for the putative non-Newtonian gradient in the borehole.

We have not been able to perform an exactly similar test on the Hilton mine experiment¹⁻³ because the surface gravity measurements have not been published. We may ask, however, what magnitude of density variations might produce the

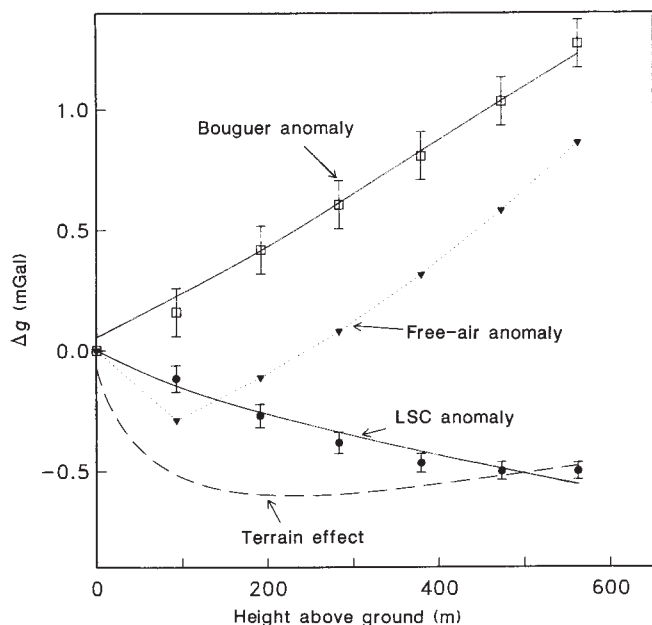


FIG. 1 Gravity anomalies on the WTVTD tower: the triangles are the observations corrected only for elevation and Earth rotation. The dashed curve is the attraction of the topography, with an assumed density of 2.67 g cm^{-3} . The LSC anomaly displays the difference between the observed and upward-continued field calculated by Romaidis *et al.*⁵ using LSC (method 1 of ref. 5). They attributed the difference to a failure of Newton's law. The accompanying solid curve is a newtonian prediction based on an incremental density contrast of 0.09 g cm^{-3} (calculation 1 of this paper). The Bouguer anomaly includes the corrections for the effect of the topography; it is the difference between the free-air anomaly and the terrain effect. The accompanying solid curve is the newtonian field of the body shown in Fig. 3 (calculation 2).

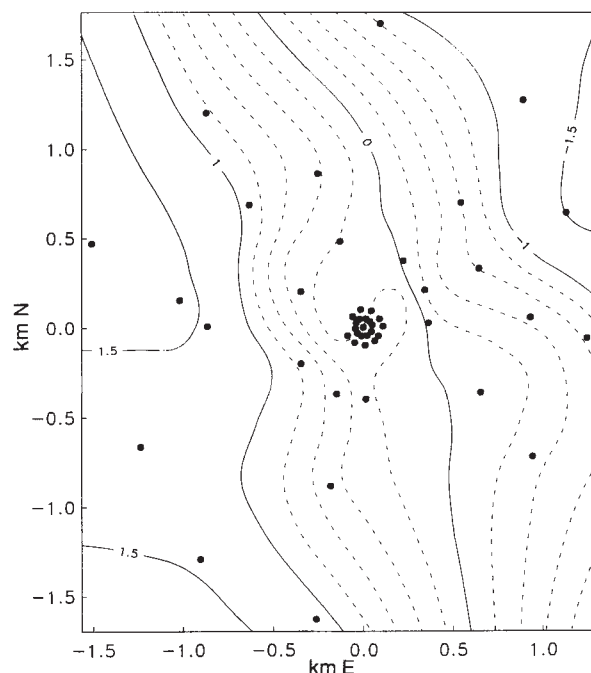


FIG. 2 Map showing the locations of 48 surface gravity stations around the WTVTD tower (at 0,0). The Bouguer-corrected gravity field (including the terrain correction) is contoured in mGal. Contours of the USGS 1:24,000 topographic map were digitized onto a grid with 33-m interval in a 10-km square around the WTVTD tower, and used to compute the newtonian attraction at all the measurement sites of the surface material, assuming a value of 2.67 g cm^{-3} for the rock density.

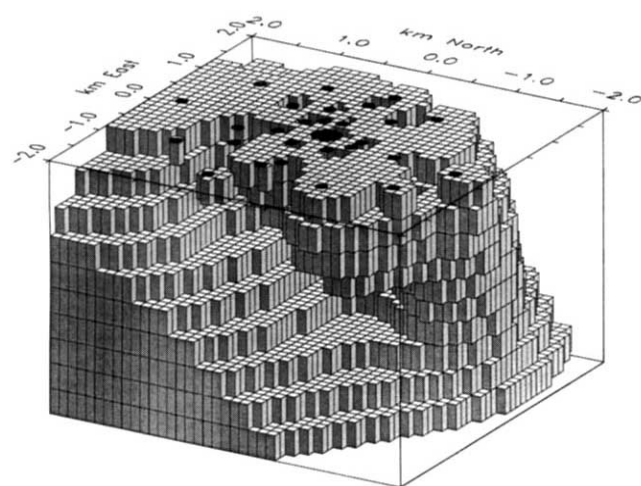


FIG. 3 One possible mass distribution accounting for the Bouguer-corrected data of Fig. 2 and the seven data shown in the topmost curve of Fig. 1. The density of the shaded cells is 0.17 g cm^{-3} less than that of the host rock. The ideal-body inversion fitted 54 gravity observations using 16,000 cells and required 20 min on a Cray XMP computer.

anomalous vertical gradient of 2 mGal km^{-1} reported in ref. 1. If variations are confined to lie below 1,500 m depth, in a volume not subjected to extensive sampling², then a density contrast of 0.12 g cm^{-3} is sufficient to produce the observed effect to an accuracy of $\sim 0.05 \text{ mGal}$. The strata of the region show jumps in density that are larger than this by a factor of 2.5 (ref. 2) and hence such a magnitude is not unreasonable. Admittedly, in this calculation we cannot reconcile our model field with the surface survey because the latter is not available; the inclusion of such observations could make a crucial difference. The only published attempt³ to incorporate that essential information into the analysis has been an estimate of the vertical gradient of g at the surface by means of an integral; values down the shaft and the associated uncertainty estimates have not yet been published.

Application to the tower experiment

Calculation 1. According to ref. 5, the differences between the LSC predictions and the observed values on the tower are due to a small, non-newtonian force. Using ideal-body theory we found the smallest density perturbation required underground to reproduce, by newtonian attraction, the supposedly non-newtonian field. To satisfy the observational constraints, the model produces an attraction that is zero (within 0.005 mGal) at the surface sites of ref. 5 and closely matches the discrepancies on the tower; see Fig. 1. The density contrast needed is only 0.09 g cm^{-3} . In other words, we have found a distribution of mass that accounts for the anomaly on the tower without disturbing the ground observations. It should be understood that this density perturbation in addition to the density variations implicitly demanded by the LSC analysis. Because of the non-uniqueness of the inverse problem, our model is just one of an infinite-dimensional family of quite different distributions with approximately the same magnitude, each capable of explaining the observations with Newton's law. The fact that the additional density changes are so small makes the demand for new physics unwarranted; had density variations of 1 g cm^{-3} been required, most geologists would have found that improbable.

Calculation 2. In their calculations, Romaides *et al.*⁵ upward-continued the observations corrected only for elevation and Earth rotation (the free-air anomalies¹⁸). Bartlett and Tew¹⁹ point out that short-wavelength gravity signals due to the local topography may not be adequately sampled in the surface gravity survey, with the consequence that the upward-continued field could be biased. In our second calculation, we included corrections for the attraction of the topography (full Bouguer

corrections¹⁸). The removal of the effect of the surface attractions from the observations on the tower gave rise to an important result: the corrected field has an almost exactly constant vertical gradient (Fig. 1). The obvious interpretation of such a field is that it is the natural vertical expression of the regional Bouguer anomaly with its deep origin, free of any controversial aspects whatever. Near geological bodies one expects the horizontal and vertical gravity gradients to be quite similar in magnitude. Horizontal Bouguer gradients in the region (Fig. 2) are typically $\sim 1.5 \text{ mGal km}^{-1}$, caused by the Northeastern Piedmont batholith in contact with the Coastal Plain deposits²⁰.

We then constructed an underground mass distribution consistent with the fully corrected gravity measurements. An ideal-body model of the Bouguer anomaly verifies the simple picture above: we find a large block of material extending to depths of 2.5 km with a density contrast of -0.17 g cm^{-3} (see Fig. 3). The surface observations were fitted to within 0.1 mGal ; a larger tolerance must be allowed as additional uncertainties have been introduced with the Bouguer correction from the unknown variation of soil cover thickness and imprecise knowledge of the density of the surface rock. Our model produces an interpolating harmonic field just like the expansion described earlier; evaluation on surface profiles around the tower show the interpolating field to be free of wild oscillations and to lie generally within 1 mGal of the mean regional gradient. This is to be expected considering the low density of the disturbing underground mass.

For computational reasons, both of the ideal-body calculations were limited to a region of $\sim 2 \text{ km}$ in radius, centred on the tower. Because of this the attraction of the finite ideal body caused an artificial gravity offset in the data near the tower relative to the distant data; this offset was 1.7 mGal for calculation 1. We found that, because they were so widely spaced, the more distant data could be offset as well by appropriate emplacement of compact masses (of ideal-body density) that had insignificant effects on the gravity values within 2 km . Because such low-density masses can produce a constant offset in the outer data without significantly affecting the inner data, it was unnecessary to include the distant data in the ideal-body calculations.

The more significant result of calculation 2 was not the ideal body itself but the intermediate result needed to obtain it; that is, the correction for terrain effects which removed the curvature in the anomaly. The ideal body in our second calculation provides an illustration of the kind of massive density anomaly required to produce large regional gravity gradients. Including a larger region in the analysis might in fact require a larger density (and would certainly require a larger computing facility) to account for the ground and tower observations, because of the Bouguer anomalies that exist in the region. The results of our first calculation, however, assure us that the increase in density would be only slight.

Conclusions

In summary, we believe that there is no compelling evidence for non-newtonian long-range forces in the three most widely cited geophysical experiments; in two of them, the Greenland experiment and the North Carolina television-mast experiments, we have shown that the gravitational fields of underground density variations with plausible magnitudes can reproduce the effects formerly ascribed to new forces. The Australian experiment has not been subjected to a sufficiently stringent test. It is important to understand that we have not shown the inverse square law to be accurate on the scales of these experiments; we have only shown that the case for its failure has not been established. More sensitive geophysical experiments might still uncover some inadequacy. Two possibilities stand out as particularly worth pursuing: an experiment on a tower that stands on a level sedimentary plane, and a marine experiment in very deep water²¹. □

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1. Stacey, F. D. & Tuck, G. J. *Nature* **292**, 230–232 (1981).
2. Holding, S. C. & Tuck, G. J. *Nature* **307**, 714–716 (1984).
3. Stacey, F. D., Tuck, G. J. & Moore, G. I. *J. geophys. Res.* **93**, 10575–10587 (1988).
4. Eckhardt, D. H., Jekeli, C., Lazarewicz, A. R., Romaides, A. J. & Sands, R. W. *Phys. Rev. Lett.* **60**, 2567–2570 (1988).
5. Romaides, A. J., Jekeli, C., Lazarewicz, A. R., Eckhardt, D. H. & Sands, R. W. *J. geophys. Res.* **94**, 1563–1572 (1989).
6. Ander, M. E. *et al. Phys. Rev. Lett.* **62**, 985–988 (1989).
7. Goldman, T., Hughes, R. J. & Nieto, M. M. *Phys. Rev. D* **36**, 1254–1256 (1987).
8. Fischbach, E., Sudarsky, D., Szafer, A., Talmadge, C. & Aronson, S. H. *Phys. Rev. Lett.* **56**, 3–6 (1986).
9. Kellogg, O. D. *Foundations of Potential Theory* (Dover, New York, 1953).
10. Tikhonov, A. N. & Afsenin, V. Y. *Solutions of Ill-posed Problems* (Wiley, New York, 1974).
11. Tikhonov, A. N., Glasko, V. B., Litvinenko, O. K. & Melikhov, V. P. *Akad. Nauk. S.S.S.R. Izv. Phys. solid Earth* **12**, 30–48; English translation 738–747 (1968).
12. Moritz, H. *Advanced Physical Geodesy* (Abacus, Tunbridge Wells, 1980).
13. Parker, R. L. *Geophys. J. R. astr. Soc.* **42**, 315–334 (1975).
14. Ander, M. E. & Huestis, S. P. *Geophysics* **52**, 1265–1278 (1987).
15. Luenberger, D. G. *Optimization by Vector Space Methods* (Wiley, New York, 1969).
16. Gill, P. E., Murray, W. & Wright, M. H. *Practical Optimization* (Academic, New York, 1981).
17. Stark, P. B. & Parker, R. L. *J. geophys. Res.* **92**, 2713–2719 (1987).
18. Garland, G. D. *The Earth's Shape and Gravity* (Pergamon, Oxford, 1977).
19. Bartlett, D. F. & Tew, W. I. *Pap. presented at Fall Meet. Am. geophys. Un., San Francisco, December* (1989).
20. Grover, D. P. thesis, Univ. North Carolina Chapel Hill (1963).
21. Hildebrand, J. A. *et al. Eos* **69**, 779–780 (1988).

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Primary structure and functional expression of the inositol 1,4,5-trisphosphate-binding protein P₄₀₀

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Cloning and expression of functional P₄₀₀ protein from cerebellar Purkinje neurons shows that this protein is a receptor for inositol 1,4,5-trisphosphate, a second messenger that mediates the release of intracellular calcium.

THE mouse cerebellum contains five different neuronal cell types. One of these, the Purkinje cell, is considered to play a central role in information processing, because it is the target of all inputs and is the only neuron that sends outputs from the cerebellar cortex. In the course of a study on cerebellar ataxic mutant mice, a membrane glycoprotein P₄₀₀ was found to be enriched in Purkinje cells from normal mice but reduced in the cerebella of Purkinje-cell-deficient mutants^{1–3}. The P₄₀₀ protein has a relative molecular mass (M_r) of 250,000 (250K)^{1,3} and is phosphorylated⁴ in a cyclic AMP-dependent manner⁵. A pulse labelling study with [¹⁴C]leucine showed that it is synthesized in the cerebellum⁴. We have solubilized and purified P₄₀₀ protein from mouse cerebella and obtained three independent anti-P₄₀₀ monoclonal antibodies^{6,7}. Immunohistochemical study of the cerebellum shows that P₄₀₀ protein is localized to the dendrites and axons, as well as cell bodies of Purkinje cells⁷, where immunoreactivity to the protein is localized on the plasma membrane, endoplasmic reticulum (ER) and postsynaptic density of Purkinje cells⁷. Western blot analysis in several other tissues reveals the presence of a small amount of P₄₀₀ protein⁶, indicating that it plays an essential part in a general membrane function that is increased in cerebellar Purkinje cells.

Inositol 1,4,5-trisphosphate (InsP₃) is known to play an important second messenger role in many cells, including neurons⁸. Recently Supattapone *et al.*⁹ purified an InsP₃-binding protein (M_r , 260K) from the rat cerebellum. This receptor is a glycoprotein capable of binding to concanavalin A and is a substrate for cAMP-dependent protein kinase^{9,10}. They also showed that [³H]InsP₃-binding activity is very much higher in the cerebellum than in any other brain area^{11,12}, and is selectively localized to Purkinje cells¹². From its characteristics (M_r , glycosylation, cAMP-dependent phosphorylation and tissue con-

centration), they propose that this receptor protein is identical to the three other cerebellar phosphoproteins—including P₄₀₀—that have been recently described^{9,13,14}. We have also confirmed that P₄₀₀ protein is identical to the InsP₃ receptor protein¹⁵.

We report here the cloning of the P₄₀₀ complementary DNA together with its amino-acid sequence deduced from the cDNA sequence¹⁶. Expression of P₄₀₀ messenger RNA was examined in the cerebellar mutant mice by northern blot analysis and was localized by *in situ* hybridization in cerebellar sections. We demonstrate that NG108-15 cells (mouse neuroblastoma/rat glioma hybrids) transfected with P₄₀₀ cDNA express binding sites for InsP₃ and that the predicted structure of the P₄₀₀ protein resembles that of the ryanodine receptor¹⁷, which mediates Ca²⁺ release from the sarcoplasmic reticulum (SR) of skeletal muscle during excitation-contraction coupling.

Cloning of P₄₀₀ cDNA

Two mouse cerebellum cDNA libraries, synthesized by priming with random hexamer or oligo(dT), were constructed in phage-λgt11 expression vectors (Fig. 1). The library was first screened with the three anti-P₄₀₀ monoclonal antibodies (4C11, 10A6 and 18A10)^{6,7}. Of 21 immunopositive candidates obtained, five were selected for further study on the basis of their strong immunoreactivity in western blot analysis (data not shown). In northern blot analysis of mouse cerebellar poly(A)⁺RNA, the cDNA inserts in all five independent clones hybridized to an mRNA of ~10 kilobases (kb), which is long enough to encode the P₄₀₀ protein (data not shown). To obtain more complete clones, we rescreened both the random-primed and oligo(dT)-primed libraries by plaque hybridization with the ³²P-labelled cDNA inserts. Two types of clone were obtained; these differed at the polyadenylation sites by 800 bases.

The complete cDNA sequence was determined¹⁶ by sequencing the 12 clones listed in Fig. 1a. There is a single large open reading frame¹⁶. Although the neighbouring sequence (CGGACATGT) of the first ATG shows only modest similarity to the initiation consensus sequence (CCA/GCCATG(G))¹⁸, it is closer to the consensus sequence than the sequence (ACAAAATGT) surrounding the second ATG. The open reading frame continues for 8,247 bases when an in-frame TAG

termination codon occurs at nucleotide position 8,248 from the first ATG. This frame was confirmed by sequencing the fusion points between the β -galactosidase gene and cDNA inserts in the five immunopositive clones tested. The sequence of the long type cDNA is 9,871 nucleotides, consisting of a 328 base pairs (bp) 5' untranslated region, a 8,247 bp coding region and a 1,296 bp 3' untranslated region which includes a polyadenylation signal (AATAAA) 14 bp upstream of a 23 base poly(A) tract. The short type cDNA is 9,071 nucleotides long, differing only in the 3' untranslated region (496 nucleotides, including AATAAA 12 bp upstream of a 23-base poly(A) tract). It seems that the long cDNA was derived from the principal transcript, as five out of the seven 3' clones, primed by oligo(dT), were of the long type.

Structure of the P₄₀₀ protein

On the basis of the cDNA sequence, the P₄₀₀ protein is predicted to comprise 2,749 amino acids and have an M_r of 313K. Although glycosylation is known to contribute to the molecular weight only slightly, this is somewhat greater than the SDS-gel electrophoresis estimate of 250K. This discrepancy may be accounted for by post-translational proteolytic processing, or aberrant electrophoretic migration due to its large size. The amino-acid composition of the predicted polypeptide agrees with that obtained by amino-acid analysis of hydrolysed P₄₀₀ (data not shown). From sequence analysis of immunopositive clones, the amino-acid sequences containing epitopes for the antibodies 4C11, 10A6 and 18A10 are localized from position 679-727, 943-1,237 and 2,648-2,749 (C-terminal end), respectively (Figs 1b and 2a).

A hydropathy profile of the protein sequence shows the existence of several stretches of hydrophobic amino acids, which could represent multiple membrane-spanning sequences (Fig.

1b and 2). Although P₄₀₀ is thought to be an integral membrane protein^{6,7}, it lacks a definable signal sequence with a hydrophobic stretch at the N-terminus, which indicates that an N-terminal hydrophilic region is exposed on the cytoplasmic face of the membrane^{17,19}. By scanning the sequence for hydrophobic stretches²⁰, nine candidates membrane-spanning regions were detected (a-i in Fig. 2a). Preliminary results from an electron microscopy study of metallo-labelled antibodies indicate that the epitopes for 4C11 and 10A6, which recognize the N-terminal region and the central region, respectively, are located to the cytoplasmic side, whereas an 18A10 epitope, which recognizes the C-terminus, is on the luminal side of the ER (N.M. *et al.*, unpublished data). The first two segments (a and b) are, therefore, unlikely to be membrane-spanning. Of the seven remaining segments (c to i), clustering in the C-terminus from amino-acid positions 2,276 to 2,589, all (or some) of the segments presumably form transmembrane α -helices and may traverse either the ER or plasma membranes of cerebellar Purkinje cells an odd number of times (see Fig. 2 legend). Consequently, the large hydrophilic N-terminal domain is exposed on the cytoplasmic face of the membrane, whereas the short C-terminal domain is oriented towards the ER lumen or outside the cell (Fig. 2b).

The protein has 21 consensus sequences for N-linked glycosylation. Most of these, however, are not involved in glycosylation, because digestion of P₄₀₀ protein with endo- β -N-acetylglucosaminidase F causes only small changes in the electrophoretic mobility⁶. Partial digestion of P₄₀₀ by *Staphylococcus aureus* V8 protease generated a polypeptide of M_r ~40K, possessing both binding activity to concanavalin A and immunoreactivity with the C-terminal-specific antibody 18A10 (data not shown). This result suggests that at least the C-terminal region is glycosylated. Additionally, the large cytoplasmic domain contains two sites that could act as targets for serine

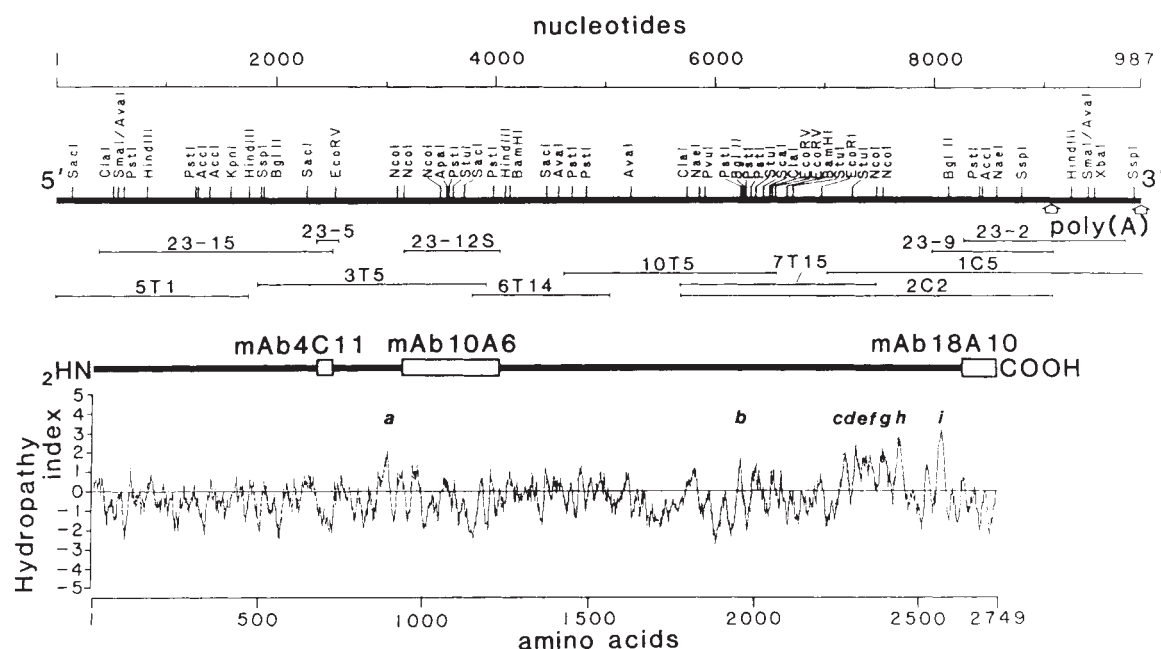


FIG. 1 a, Restriction map of the P₄₀₀ cDNA clones. The positions of the 12 overlapping clones are indicated. Two open arrows point to the polyadenylation sites. b, Hydropathy plot of protein-coding region. Three open boxes represent the regions containing epitope sequences for monoclonal antibodies (mAb) 4C11, 10A6 and 18A10. Hydrophobic segments (a to i) are indicated.

METHODS. Poly(A)⁺RNA was isolated from cerebella of two- to three-week-old mice (ICR) by the guanidinium-CsCl method³⁶, followed by purification by oligo(dT)-cellulose chromatography³⁶. The cDNA was synthesized using a random hexamer or oligo(dT)₁₂₋₁₈ as primers essentially as described previously³⁷. For random-primed cDNA synthesis, poly(A)⁺RNA with a sedimentation coefficient of more than 23S in sucrose gradients (15–30%

sucrose gradients spun at 30,000 r.p.m. in a Beckman SW41Ti rotor for 16 h at 25 °C) was used. Both random-primed and oligo(dT)-primed cDNAs were ligated in the lambda-gt11 expression vector³⁸. First, the random-primed library was immunoscreened for the production of fusion protein containing putative P₄₀₀ epitopes in the host *E. coli* strain Y1090r³⁸ using monoclonal antibodies^{38,6}. At subsequent stages, both the random-primed and oligo(dT)-primed libraries were screened by plaque hybridization using the ³²P-labelled cDNA inserts derived from immunopositive clones (clone 23-2, 23-5, 23-9, 23-12S and 23-15 in Fig. 1a). The cDNA inserts were subcloned into pUC118 or pUC119 (ref. 39) and sequenced¹⁶ by the dideoxy chain termination method⁴⁰. The hydropathy plot was computed according to Kyte and Doolittle⁴¹; the window size is 17 residues.

a 1 MSDKMSSFLHIGDICSLEYAEGSTNGFISTLGLVDDRCVVÖPEAGDLNNPÖKKFRDCLFKLCPMNRYSAQÖQFWKAAPGÄNSTTDAVLLNKLHHAADLEK 100
 101 KQNETENRKLLGTVIOYGNVÖIQLHLKSNKYLTIVNKRPLÄLEKNAMRVÖLDEAGNEGSWFYIQPFYKLRÖSIGDSVVIÖGÖVVLNPNVNAÖQPLHASSHÖL 200
 201 VDNPGCNEVNSVNCNTSWKÖIVLFMKWSDNÖDDILKGGDVÖRFLHAEQEFÖLTCDEHRKKÖHVFLRTTGRÖSATSSÖKÄLWEVEVVOHÖPCRGGAGYWN 300
 301 SLFRFKHLAÖGHYLAEEVÖDÖFEEECLEFÖQPSVDPDÖDÄSRÖRLNAQÖKMVYSLVSPÖGNDISSIFÖLDPTTLRGGÖSLVPRNSYVRÖLRHLCÖTNTWVH 400
 401 STNIPIDKEÖEÖKPVMKIGÖISPLKEDKEAFÖIVPVSPAÖVÖRDLÖFANDASÖKVLGSIAGKÖLEKGTITQNEÖRRSVTKLLEDÖLVYFVTGGTNSÖGÖDVLEVVFÖS 500
 501 KPNRERQKLMÖREONILKQIFÖKLLQAPFÖTÖCGDGPMLRLEÖELGDORHAPFRÖHICRLCYRVÖLRHSQÖDYRKÖNÖEYIAKQFÖGFMQKQIGYDVÖLÄEDTITALLH 600
 601 NNRKLEKHÖITAAEIDTFVÖSLVRKNRÖPRFÖLDYLSÖLCVÖSMNKSIPVÖTÖELICKAVLNÖPINADILIEÖTKÖVLSRFEFEGÖVSTGENALEAÖGEDEEEVWLFW 700
 701 RDSNKEIRÖSKSVRELAQDAÖEGQKEDRDILÖSYRYQLNÖLFARMCLDRQYLAÖINEISGÖLÖVÖDLILRÖCMSÖDENLPYDLRASFÖCRLMLHMVÖDRDPQÖQVTP 800
 801 VKYARLWSEIPSEIAIDDYÖSSGTSKDEIKÖERFAQTMÖFVÖEYLRDVVCÖRFPFSDÖKKNKLTÖFEVVLÄRNLIFÖYGFYNSÖDLLRLTKILLÄILDCVHÖV 900
 901 TITFIPISÖKMÖKGEENKGSNÖMRSIHGÖVGEÖLMTQVVLRGGÖFLPMTMAAÖPEGNVQAEÖPEKEDIMVMDÖTKLIIÖILOQÖILNVRLDYRÖISCLLCIFKRÖE 1000
 1001 FDESNSQSSÖETSSGNSÖQÖGPSNVPGALDÖFHEIEQAEGÖIFGGSEENTPÖLDLDHGGRTÖFLRVLLHÖLTMHÖYPPLVSGAÖLQÖLFRHFSÖQRÖEVLOAFKÖV 1100
 1101 OLLVTSODVÖNYKQIKÖDLÖLRSIVEKÖSÖLWVYKGGQDÖPEMDGASGÖNEHKKTEEGTÖSKPLKHESTÖSSÖNYRVVKEILÖIRLSKLCVÖQÖESASVRKSRKÖ 1200
 1201 QORLLRNMGÄHAVVLELLQÖIPYEAÖDTKMÖEIMRLAHEÖFLQNFCAQÖNQÖALLKHÖINÖFLKPGILEÄVTMQHIFMNNÖFQLCSEINÖERVVOHFVHCÖE 1300
 1301 THGRNVQYIKÖFLOTIVKAEÖGKÖIKKQDMVMAELVNSGÖDVLFYNDRAÖSFQTLQMMRÖSERDRMDENSÖPLMYHIHLVÖELAVCTEGKNVÖYTEIKCNSLÖ 1400
 1401 PLDDIVRVVÖIHEDCIPÖVKIÖIAYINFLNHCYÖVDETEVEMKÖIÖYTSNHMWKLFENFLVDICRÄCÖNNTSDRKHÄDSILEKYVÖTEIVMSIVTTFÖSSPFSÖQSTI 1500
 1501 LOTROPVFVÖLLQGVFRVYÖHCNWLMPÖSQKÄSVESCIRVLSÖDVAKSRAIÖIPVDLDSQVNNÖFLKSHNIVÖKTALNWRLSÄRNAARRDSVÖLÄASRDYRNII 1600
 1601 ERLQDIVSÄLEDRLRPLVQÄELSVLDVVLHÖRPELLFENÖIDARRKCESGÖGÖICKLIKHTÖKÖLLEENEEKLÖCKIKVLOTLRÖMTKDRGYGÖEKOISIDÖSEN 1700
 1701 AELPQAPEÄENSTEÖELEPÖSPPLRÖLEDHÖKRGEALRÖILVÖNRYGNIÖRPSÖGRRESLTSFÖGNGPLSPGGPÖSKPGGGGGPÖSSSTSRGEMÖSLAEVOCHLDK 1800
 1801 EGASNLVIDÖIMNASSDRVÖFHESILLAIÖALEGGNTTIÖHÖSFFCRLTEDÖKKSEKFFKVFÖYDRMKVAQÖEIKATVTVNTSÖLGNKKKDDÖVDRDAPSÖRKKÄ 1900
 1901 KEPTTQITÖEÖVRDQÖLLEASÄATRKAFITÖFREADPDÖDHYÖSGEGTQATTÖKAKDDLEMSÄVITIMQPIÖLRÖLQÖLCCENHÖNRÖLQNLFRÖCÖNNKNTYNLVC 2000
 2001 ETLOFLDCIÖGÖSTTGGGLGÖGLYINEKNVÄLINQÖTLESÖLÖTEYCOGÖPCHEÖNÖNCIATHESÖNGÖIDIITÄLILÖNDINPLGKKÖRMDLVLELKNÖNASKLLLAÖIMÖ 2100
 2101 SRHDSÖNAERÖILYNMRPKÖLÖVEVÖKKAYMÖGEVEFEDGEÖNGEDGAASPRNÖVGHNIYILAHÖLARHNKELÖTMLKPGGQVÖDÖGEALEFYAKHTAQIEIVRÖL 2200
 2201 DRTMEOIVFPVPSICEFLTKÖESKLRIÖYTTIERDEOGSKINÖDFLRSEDÖLFNEMNWQKLRÄQPVLYWCÄRNMÖSFWSSISFNLAVLMNLLVÄFFYPFKGVÖR 2300
 2301 GGTLEPHWSÖGLWTAMLISÄIVIALPKPÖHGIRALIASTILRLIFSÖVGLÖPTFLFLGAFNÖVCNKIIFLMÖSFVGNCGTFTÖRGYAMVLDVÖFLYHLLYLLI 2400
 2401 CAMGLFVHEÖFFYSLLFDLÖVYREÖTLLNVIÖKSVTRNGRSIÖILTAVLALILVYLFISVIGYLFÖFKDDFILEVÖDRLPNETAVPÖETGESLANDÖFLYSDVCRVÖE 2500
 2501 GENCTSPAPKEÖELLPAÖETÖQÖKEHTCETÖLÖMCIÖTVLSHÖLRSGGVGÖVLRKPSKEÖPLFAARVIOYDÖLFFFMVIOIÖVLNIFGVIÖDÖTFADLRSEKÖ 2600
 2601 KKEEILKTTÖCFICGLERDKFÖDNKTVIFEEHÖIKEEHNMWHYLCFIVLVKÖVÖDSTEYTGPEÖSYVAEMIRÖNLDWFLMRÄMSLVSSDSEGÖEQNELRNLOEK 2700
 2701 LESTMKLVTNLSGÖLSELKÖDMTEQRKÖKÖRIÖGLLGHPPHNMVNPOÖPA 2749

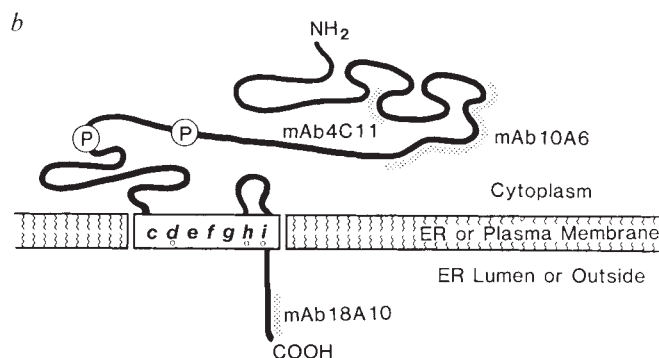


FIG. 2 Predicted amino-acid sequence and structural model of P_{400} protein. *a*, The amino-acid sequence of P_{400} protein was deduced from its cDNA sequence¹⁶ (EMBL database accession number X15373). The regions containing epitope sequences for monoclonal antibodies are shown by dashed lines. The proposed membrane-spanning sequences are represented by double dashed lines (a-i). The filled arrowheads mark the potential phosphorylation sites for cAMP-dependent protein kinase. The 3'-deletion construct shown in Fig. 5 encodes a polypeptide from the first methionine to the cysteine of position 2,215, marked with the open arrowhead. *b*, A schematic model for the topology of P_{400} protein in the membrane. A computer program (ALOM), for prediction of membrane-spanning segments²⁰, in the Integrated Database and Extended Analysis System (IDEAS) was used through the National Institute of Genetics (NIG) in Japan. Possibilities for membrane-spanning become confident in the following orders: segments i>h>d>g>a>c>f>e>b (peripheral:integral odds = 7.0E-07 > 8.1E-06 > 5.1E-04 > 4.5E-03 > 1.2E-02 > 3.2E-02 > 7.8E-02 > 4.0E-01 > 5.3E-01, respectively). To orient termini to opposite sides, the P_{400} protein should traverse the membrane an odd number of times. Epitopes for antibodies 4C11 and 10A6 are localized to the cytoplasmic side, indicating that segment a is not membrane-spanning. We assume, therefore, that P_{400} traverses the membrane at most seven times (c to i) and at least 3 times because of the presence of three reliable segments d, h and i with high odds. Based on this prediction and the epitope localizations, we have designed the structural model for P_{400} as a membrane-spanning protein. Putative transmembrane domains are indicated by an open box. The C-terminal domain is either located in the ER lumen or outside the cell by anchoring to the ER and plasma membrane, respectively. The regions containing epitope sequences for monoclonal antibodies are shown by dotted lines. The potential phosphorylation sites are represented by an encircled P.

phosphorylation (amino acids 1,588 and 1,755), a possibility supported by the fact that a serine residue of P_{400} protein is known to be phosphorylated in a cAMP-dependent manner⁵.

P_{400} expression in Purkinje cells

In situ hybridization histochemistry clearly shows that P_{400} mRNA is localized in the perikaryon of Purkinje cells of the cerebellum (Fig. 3). We examined gene expression of P_{400} in the cerebellar ataxic mice by northern blot analysis (Fig. 4). The *staggerer* mutant Purkinje cells have a withered dendritic arbor lacking the tertiary branchlet spines that serve as the sites of

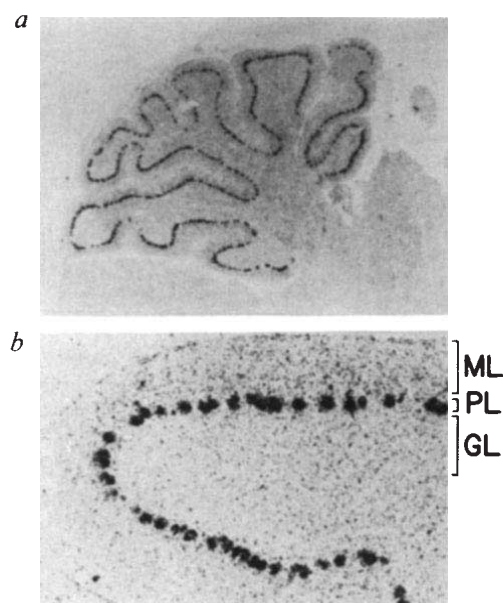


FIG. 3 Localization of the P_{400} -specific mRNA by *in situ* hybridization. *a*, Autoradiograph of a sagittal section of the cerebellum. *b*, Higher magnification of *a*. ML, molecular layer; PL, Purkinje cell layer; GL, granular layer. Sections (6 μ m) in paraffin were prepared from the cerebellum of 30-day-old ICR mice. *In situ* hybridization with a random-primed ³⁵S-labelled P_{400} cDNA was as before⁴².

synapse between the granule cells and Purkinje cells²¹. The disorder of Purkinje cells is most severe in the *Purkinje cell degeneration* (*pcd*) and *nervous* mutants, where most of these cells degenerate selectively (95–99% in *pcd*²² and about 85% in *nervous*²³). The cerebella of these Purkinje cell-deficient mutants contain P_{400} mRNA at extremely low levels, as expected (lanes 2, 3 and 4 in Fig. 4). On the other hand, the cerebellum of the *weaver* mutant, which lacks granule cells with no significant loss of other cerebellar neurons²⁴, contains an increased amount of P_{400} mRNA compared with the wild-type cerebellum (lane 4). This increase in the *weaver* mutant was also observed at the protein level^{1,4} and is caused by a relative increase in the proportion of Purkinje cells in the cerebellum, due to the dearth of granule cells.

These results clearly indicate a predominant distribution of P_{400} gene expression in the cerebellar Purkinje cells. Our preliminary experiments, however, have shown that the expression of P_{400} mRNA can be detected at a level of about one fiftieth in the cerebrum and at much lower levels in other tissues (data not shown), indicating that P_{400} expression is widespread and possibly general.

InsP₃-binding properties

Our recent results indicate that P_{400} protein has InsP₃-binding activity¹⁵. To examine whether the P_{400} cDNA clone encodes an InsP₃-binding protein, we performed InsP₃-binding assays on protein expressed in the neuroblastoma/glioma hybrid cell line NG108-15. In the neuron-like NG108-15 cells, it is known that InsP₃ acts as a second messenger and transiently raises intracellular Ca²⁺ (ref. 25). This cell contains an endogenous protein immunoreactive to the anti- P_{400} monoclonal antibody 4C11 (Fig. 5*a*, lane 4). This protein cross-reacts equally with all three of our monoclonal antibodies (data not shown). It is interesting that the molecular size of the endogenous P_{400} protein

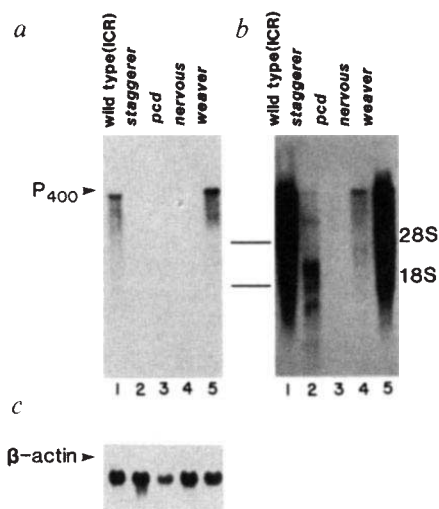


FIG. 4 Northern blot analysis of P_{400} mRNA in the cerebella of mutant mice. *a*, Wild-type ICR, lane 1; *staggerer*, lane 2; *pcd*, lane 3; *nervous*, lane 4; and *weaver*, lane 5 mice. *b*, Long exposure of the hybridization membrane in *a*. *c*, Rehybridization of the same blot in *a* with a β -actin probe to assess the relative amounts of RNA in each lane. Although an equal amount of total RNA was used, *pcd* showed a weaker signal for β -actin mRNA.

METHODS. Total cellular RNA (20 μ g) from the cerebella was electrophoresed on a 1% agarose-formaldehyde gel³⁶, blotted to a nylon membrane (Biodyne A, Pall) and hybridized at 42 °C for 20 h with random-primed ³²P-labelled P_{400} cDNA or β -actin cDNA probes (1×10^3 c.p.m. μ g⁻¹, $1-2 \times 10^6$ c.p.m. ml⁻¹) in 50% formamide, 5 $\times$ Denhardt's solution, 5 $\times$ SSC, 50 mM sodium phosphate (pH 6.5), 0.1% SDS, 100 μ g ml⁻¹ heat-denatured herring sperm DNA and 10% dextran sulphate. The membrane was washed twice in 2 $\times$ SSC and 0.1% SDS at room temperature for 10 min, twice in 0.1 $\times$ SSC and 0.1% SDS at 65 °C for 30 min. The blot was exposed at -70 °C to X-ray film with an intensifying screen.

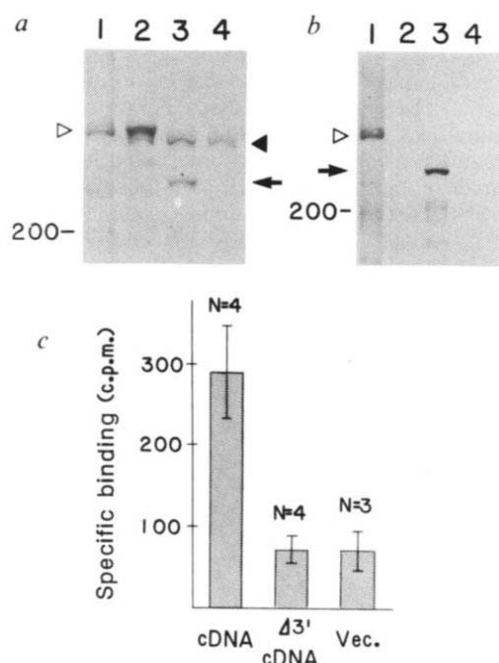


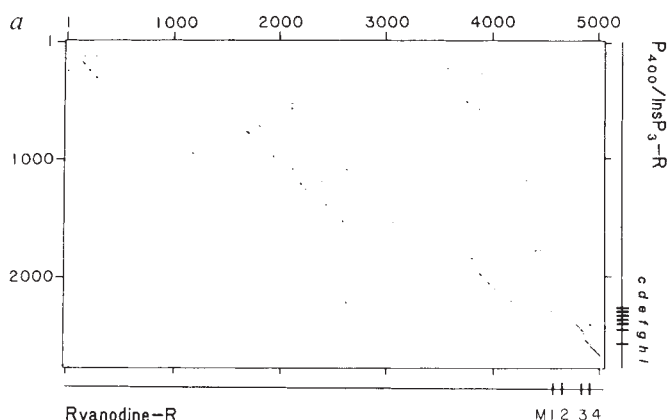
FIG. 5 Binding of [^3H]InsP $_3$ to P $_{400}$ protein expressed in NG108-15 cells. *a*, Western blot analysis of crude membrane proteins using the anti-P $_{400}$ monoclonal antibody 4C11. Lane 1, Microsomal proteins (5 μg) prepared from the mouse cerebellum; lane 2, crude membrane proteins (50 μg) prepared from the NG108-15 transfected with P $_{400}$ cDNA; lane 3, 3'-deletion construct and lane 4, vector plasmid DNA. Cerebellar P $_{400}$ protein (Δ); endogenous P $_{400}$ of NG108-15 ($\blacktriangle$); truncated P $_{400}$ ($\rightarrow$). The position is shown of a molecular weight marker ($\times 10^{-3}$). *b*, Western blot analysis of soluble cytosolic proteins using 4C11. Lane 1, the same as lane 1 of *a*; lanes 2 to 4, soluble cytosolic proteins (15 μg) prepared from the same cells as those in lanes 2 to 4 of *a*, respectively. *c*, Specific binding of [^3H]InsP $_3$ to crude membrane proteins prepared from NG108-15 transfected with P $_{400}$ cDNA (cDNA), the 3'-deletion construct ($\Delta 3'$ cDNA) or vector DNA (Vec). *N* represents the number of samples tested. Results are the means $\pm$ s.e.m. of *N* samples.

METHODS. Plasmid DNAs used for the transfection assay were constructed as follows. pBact-CAT9 (ref. 43) was digested with *Hind*III and *Hpa*I to remove the chloramphenicol acetyltransferase (CAT) sequence, treated with Klenow enzyme and dNTPs, and ligated to a *Sall* linker (GGTCGACC). The resulting plasmid pBact-S, containing a single *Sall* site in place of the CAT gene fragment, between the β -actin promoter and SV40 polyadenylation sequences, was used as a vector. To generate pBact-Cl, the full P $_{400}$ cDNA (short type in Fig. 1) was ligated to *Sall* linkers at both ends and inserted into the *Sall* site of pBact-S. The DNA fragment from the 5' end to an internal *Eco*RI site (6,646 nucleotide position in Fig. 1) of P $_{400}$ cDNA was also inserted into the *Sall* site of pBact-S. The resultant plasmid pBact-EL2 encodes a $\sim 252\text{K}$ truncated polypeptide which lacks the 534 amino acids of the C-terminal portion (Fig. 2). These plasmid DNAs were introduced into NG108-15 cells by the standard calcium phosphate precipitation technique⁴⁴. Two days after transfection, cells were collected, washed with ice-cold TBS (Tris-buffered saline) and resuspended in ice-cold 0.25M sucrose in buffer A (10 mM Tris-HCl, pH 8.0, 0.1 mM EDTA, 1 mM PMSF, 0.01 mM pepstatin A, 0.2 U ml $^{-1}$ aprotinin). The cells were homogenized by 10 strokes in a chilled glass-Teflon Potter homogenizer and centrifuged at 3,300g for 3 min at 2°C to remove nuclei. The supernatants were recentrifuged at 95,000 r.p.m. ($393,201 \times g_{\text{max}}$) in a Beckman TLA100.2 rotor for 5 min at 2°C. The resulting pellets and supernatants were used as crude membrane proteins and soluble cytosolic proteins, respectively. Some of the protein was used for western blot analysis^{6,7}. Cerebellar microsome was prepared as described⁷. The [^3H]InsP $_3$ -binding assay, using rapid centrifugation¹⁵ for membrane fractionation or PEG precipitation for soluble fractionation¹⁵, was performed by incubating 50 μg of the proteins in 100 μl buffer A containing 10 nM [^3H]InsP $_3$ (NEN) for 10 min at 4°C. Nonspecific binding was determined in the presence of 1 μM cold InsP $_3$. Specific binding was defined by the subtraction of nonspecific binding, ranging from 200 to 250 c.p.m., from total binding.

in NG108-15 (lane 4) is less than that in the mouse cerebellum (lane 1). This size difference allows discrimination between cerebellar P $_{400}$ and endogenous P $_{400}$ in NG108-15. Scatchard analysis using [^3H]InsP $_3$ revealed that the NG108-15 P $_{400}$ differs from the cerebellar P $_{400}$ in having two specific binding components, of high- and low-affinity, with low capacity (A.M. *et al.*, unpublished data). In the transient assay, the P $_{400}$ cDNA-transfected cells produced a large amount of protein immunoreactive to all three of the anti-P $_{400}$ monoclonal antibodies (Fig. 5*a*, lane 2; immunoreaction pattern with 4C11). Both the cDNA-derived and endogenous P $_{400}$ proteins were observed in membrane fractions, but not in soluble cytosolic fractions (lane 2 in Fig. 5*a, b*). In the cDNA-transfected cells, expression of the cerebellar-type P $_{400}$ protein, derived from the cDNA, is apparently coupled with the elevation of [^3H]InsP $_3$ binding activity (Fig. 5*c*). The expressed protein displays a high affinity (K_D , 21.7 nM) and specificity for Ins(1,4,5)P $_3$ with high capacity, as does P $_{400}$ in cerebellar microsomes^{11,15} (A.M. *et al.*, unpublished data). According to our preliminary data, specific [^3H]Ins(1,4,5)P $_3$ binding of the expressed protein is competed out tenfold less by Ins(2,4,5)P $_3$ than by Ins(1,4,5)P $_3$. The potency order of competition by various inositol phosphates is: Ins(1,4,5)P $_3$ > Ins(2,4,5)P $_3$ $\geq$ Ins(1,3,4,5)P $_4$ > Ins(1,4)P $_2$. Ins(1)P is inactive in competition at concentrations as high as 50 μM . When transfected with the 3'-deletion construct that lacks the 3' region encoding the 534 amino-acid residues of the C-terminus, including the putative transmembrane domains (Fig. 2), NG108-15 produced a truncated protein ($\sim 252\text{K}$) immunoreactive to antibodies 4C11 and 10A6, but not 18A10. Expression of the truncated protein was not accompanied by elevation of InsP $_3$ -binding activity (Fig. 5*c*). Truncated protein was observed mostly in the soluble cytosolic fraction (lane 3 in Fig. 5*b*), about 1% being found in the membrane fraction (lane 3 in Fig. 5*a*), which could be due to contamination during preparation.

These results demonstrate that the P $_{400}$ protein encoded by our cDNA is present on membranes and does possess InsP $_3$ binding activity. The results from the transfection assay with the 3'-deletion construct reveal that C-terminal hydrophobic stretches could function as a membrane anchor, because the mutant protein accumulates in the cytoplasm. The deleted region

FIG. 6 Sequence comparison between the murine InsP $_3$ -binding protein P $_{400}$ and the rabbit ryanodine receptor. *a*, Diagonal matrix comparison using a DIAGON computer program⁴⁵ modified by Dr T. Inoue. The dots correspond to mid-points of 25-residue spans, giving a double-matching probability that the mean score is ≥ 1.7 . Solid bars indicate possible transmembrane segments. *b*, Alignment of the amino-acid sequences. Identical residues are enclosed within solid boxes and conservative substitutions within dashed boxes. Gaps (dashes) were introduced to maximize homology. The numbers of inserted residues are indicated in parentheses. *c-i*, Putative P $_{400}$ membrane-spanning regions. M1-M4, putative ryanodine receptor membrane-spanning sequences.



may also be responsible for InsP_3 binding, or may be important in forming a conformation capable of InsP_3 binding.

Discussion

Similar molecular features and spatial distributions suggest an identity between the P_{400} protein and the InsP_3 receptor/binding protein reported by Snyder *et al.*⁹⁻¹². Recently, Nordquist *et al.*²⁶ isolated cDNA clones from a library of normal mouse cerebellar cDNA by differential screening with mRNA from the cerebellum of *pcd* mutant mice. We have found that one clone, PCD6, contains a 500-amino-acid sequence which has an identity with the C-terminal region of P_{400} protein, except that the

leucine at residue 2,675 of P_{400} is a proline in PCD6. Distribution of PCD6 mRNA expression is also similar to that of P_{400} . These results indicate that PCD6 is a partial clone of P_{400} .

InsP_3 is thought to act by binding to a specific intracellular receptor, probably on a component of the ER⁸ or a more discrete specialized structure, the 'calciosome'^{27,28}. There are some discrepancies, however, between the subcellular localization described in our results⁷ and those reported by Ross *et al.*²⁹. Using polyclonal antibodies they localized the InsP_3 receptor to the ER, the subplasmalemmal cisternae and the nuclear membrane, but not to the plasma membrane. Our electron microscopy study using monoclonal antibody 4C11 indicates



that in the cerebellar Purkinje cells P_{400} protein is present at the ER, the plasma membrane and the postsynaptic density⁷. Two other antibodies also stained the plasma membrane clearly, as reported previously⁷ (N.M., unpublished data). $InsP_3$ was recently found to activate Ca^{2+} channels in the plasma membrane of T lymphocytes³⁰. This indicates that our monoclonal antibodies may efficiently react with $InsP_3$ receptors in the plasma membrane and that in Purkinje cells, $InsP_3$ may act on an $InsP_3$ -gated calcium channel in the plasma membrane, as is the case with T lymphocytes. Our data also indicate that the external, but not the internal, surface of the nuclear membrane is only weakly positive (Y. Inoue *et al.*, unpublished data), contrary to the strong immunoreaction reported by Ross *et al.*²⁹. Our three different monoclonal antibodies show almost the same localization pattern. The discrepancy between these results may therefore be due to differences in antibody specificity. This particular subcellular localization may be specific to Purkinje cells in which the P_{400} protein is extremely enriched, and together with the Ca^{2+} -mobilization system (Ca^{2+} -binding proteins, protein kinase C)^{11,12}, is associated with $InsP_3$ activity and synaptic transmission³¹.

The structural basis of $InsP_3$ binding is still unclear, although Scatchard analysis implies the existence of a single binding site¹⁵. Our structural model suggests that positively charged amino-acid residues in the large cytoplasmic N-terminal region or in the cytoplasmic loop region (Fig. 2b) may be responsible for binding negatively charged $InsP_3$ accumulated in the cytoplasmic compartment. $InsP_3$ has a role in releasing Ca^{2+} from intracellular sites such as the ER⁸. It is noteworthy that the *staggerer* Purkinje cell, defective in P_{400} production (Fig. 4), can produce sodium spikes but not calcium spikes³². It is probable that P_{400} has some affinity for Ca^{2+} and that in response to $InsP_3$ signals, the P_{400} protein itself is involved in Ca^{2+} release. We could not, however, find any apparent homologies to Ca^{2+} -binding sites such as the consensus sequence for EF-hand³³, the putative Ca^{2+} -binding region of Ca^{2+} -ATPases³⁴, or the hypothetical Ca^{2+} -binding sites in the vicinity of segment S2 of the dihydropyridine receptor¹⁹. Our recent reconstitution experiments with planar lipid bilayers, however, show that the purified P_{400} complex exhibits an $InsP_3$ -induced Ca^{2+} release (N.M. *et al.*, in preparation), which provides strong support for the iden-

tity of our P_{400} protein with the $InsP_3$ receptor.

Recently, Takeshima *et al.*¹⁷ have described the primary structure of the ryanodine receptor, which is the channel responsible for the release of Ca^{2+} from the sarcoplasmic reticulum of skeletal muscle during excitation-contraction coupling. Surprisingly, P_{400} has fragmentary sequence homology with the ryanodine receptor protein (Fig. 6a). In particular, the putative transmembrane domains h and i and the successive C-terminal region in P_{400} have remarkable similarity to the transmembrane segments M3 and M4 and the successive C-terminal region in the ryanodine receptor (Fig. 6b). This homology does not extend to the binding sites for modulators (Ca^{2+} , nucleotides, calmodulin) as proposed by Takeshima *et al.*¹⁷. Particularly striking, nevertheless, is the overall resemblance between these two receptors of the proposed transmembrane topology, both sharing a large N-terminal region located on the cytoplasmic side and a short C-terminal region spanning the membrane. Our recent electron microscopy of purified P_{400} complex revealed a square shape¹⁵ similar to that of the ryanodine receptor Ca^{2+} -release channel complex³⁵, suggesting that they share a similar tertiary structure. These observations imply related Ca^{2+} mobilization functions. In spite of these similarities, there are some differences in the structure between the ryanodine receptor¹⁷ and P_{400} . The region encompassing segments M2 and M3 of the ryanodine receptor was claimed to have some sequence homology with the M2-M3 region of the subunits of the nicotinic acetylcholine receptor, but the corresponding region of P_{400} does not. We think that this point is due to the differences in interpretation of the amino-acid sequence. Also, the ryanodine receptor was proposed to have four membrane-spanning segments, with its C-terminal region on the cytoplasmic side of the sarcoplasmic reticulum. We assume, however, that P_{400} has at least three (and at most seven) transmembrane domains and that the C-terminal is therefore in the ER lumen or outside the cell (Fig. 2b). Our model is based on immunoelectron microscopic observations using immunogold, which show that the N- and C-termini, detected with three different monoclonal antibodies, are located to the cytoplasmic and luminal sides of the ER respectively. At present, we cannot reconcile the discrepancy and infer that the difference reflects the molecular features which distinguish P_{400} from the ryanodine receptor. □

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- Mallet, J. *et al.* *Brain Res.* **103**, 291–312 (1976).
- Mikoshiba, K. & Changeux, J.-P. *Brain Res.* **142**, 487–504 (1978).
- Mikoshiba, K., Hutcheson, M. & Changeux, J.-P. *Dev. Neurosci.* **2**, 254–275 (1979).
- Mikoshiba, K., Okano, H. & Tsukada, Y. *Dev. Neurosci.* **7**, 179–187 (1985).
- Yamamoto, H. *et al.* *J. Neurochem.* **53**, 917–923 (1989).
- Maeda, N. *et al.* *J. Neurochem.* **51**, 1724–1730 (1988).
- Maeda, N. *Dev. Biol.* **133**, 67–76 (1989).
- Berridge, M. J. A. *Rev. Biochem.* **56**, 615–649 (1987).
- Supattapone, S. *et al.* *J. Biol. Chem.* **263**, 1530–1534 (1988).
- Supattapone, S. *et al.* *Proc. natn. Acad. Sci. U.S.A.* **85**, 8747–8750 (1988).
- Worley, P. F. *et al.* *Nature* **325**, 159–161 (1987).
- Worley, P. F., Baraban, J. M. & Snyder, S. H. *J. Neurosci.* **9**, 339–349 (1989).
- Groszwald, D. E. & Kelly, P. T. *J. Neurochem.* **42**, 534–546 (1984).
- Walaas, S. I., Nairn, A. C. & Greengard, P. *J. Neurosci.* **6**, 954–961 (1986).
- Maeda, N., Ninobe, M. & Mikoshiba, K. *EMBO J.* (manuscript submitted).
- Furuichi, T., Yoshikawa, S. & Mikoshiba, K. *Nucleic Acids Res.* **17**, 5385–5386 (1989).
- Takeshima, H. *et al.* *Nature* **339**, 439–445 (1989).
- Kozak, M. *Nucleic Acids Res.* **12**, 857–872 (1984).
- Tanabe, T. *et al.* *Nature* **328**, 313–318 (1987).
- Klein, P., Kanehisa, M. & DeLisi, C. *Biochim. biophys. Acta* **815**, 468–476 (1985).
- Hirano, A. & Dembitzer, H. M. *J. Neuropathol. exp. Neurol.* **34**, 1–11 (1975).
- Mullen, R. J., Eicher, E. M. & Sidman, R. J. *Proc. natn. Acad. Sci. U.S.A.* **73**, 208–212 (1976).
- Landis, S. J. *Cell Biol.* **57**, 782–797 (1973).
- Rakic, P. & Sidman, R. L. *J. Comp. Neurol.* **152**, 103–133 (1973).
- Higashida, H. & Brown, D. A. *Nature* **323**, 333–335 (1986).
- Nordquist, D. T., Kozak, C. A. & Orr, H. T. *J. Neurosci.* **8**, 4780–4789 (1989).

- Volpe, P. *et al.* *Proc. natn. Acad. Sci. U.S.A.* **85**, 1091–1095 (1988).
- Hashimoto, S. *et al.* *J. Cell Biol.* **107**, 2523–2531 (1988).
- Ross, C. A. *et al.* *Nature* **339**, 468–470 (1989).
- Kuno, M. & Gardner, P. *Nature* **326**, 301–304 (1987).
- Blackstone, C. D., Supattapone, S. & Snyder, S. H. *Proc. natn. Acad. Sci. U.S.A.* **86**, 4316–4320 (1989).
- Crepel, F., Dupont, J.-L. & Gardette, R. *J. Physiol. Lond.* **346**, 111–125 (1984).
- Perrett, C., Lomri, N. & Thomasset, M. *J. molec. Evol.* **27**, 351–364 (1988).
- Brandl, C. J. *et al.* *Cell* **44**, 597–607 (1986).
- Anderson, K. *et al.* *J. Biol. Chem.* **264**, 1329–1335 (1989).
- Maniatis, T., Fritsch, E. F. & Sambrook, J. in *Molecular Cloning, A Laboratory Manual* (Cold Spring Harbor, New York, 1982).
- Gubler, U. & Hoffman, B. J. *Gene* **25**, 263–269 (1983).
- Huynh, T. V., Young, R. A. & Davis, R. W. in *DNA Cloning, A Practical Approach* Vol. 1 (ed. Glover, D. M.) 49–78 (IRL, Oxford, 1985).
- Vieira, J. & Messing, J. in *Methods in Enzymology*, vol. 153 (eds Wu, R. & Gross) 3–11 (Academic Press, Inc. San Diego, 1987).
- Sanger, F., Nicklen, S. & Coulson, A. R. *Proc. natn. Acad. Sci. U.S.A.* **74**, 5463–5467 (1977).
- Kyte, J. & Doolittle, R. F. *J. molec. Biol.* **157**, 105–132 (1982).
- Shiota, C., Miura, M. & Mikoshiba, K. *Dev. Brain Res.* **45**, 83–94 (1989).
- Friegien, N. & Davidson, N. *Gene* **48**, 1–11 (1986).
- Miura, M. *et al.* *Gene* **75**, 31–38 (1989).
- Staden, R. *Nucleic Acids Res.* **10**, 2951–2961 (1982).

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Tyrosine phosphorylation of the fission yeast *cdc2*⁺ protein kinase regulates entry into mitosis

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The *cdc2*⁺ protein kinase (pp34) is found to be phosphorylated on tyrosine as well as serine and threonine residues in exponentially growing *Schizosaccharomyces pombe*. At mitosis, the level of pp34 phosphorylation on both threonine and tyrosine residues decreases. The single detectable site of tyrosine phosphorylation in pp34 has been mapped to Tyr 15, a residue within the presumptive ATP-binding domain. Substitution of this tyrosine by phenylalanine advances cells prematurely into mitosis, establishing that tyrosine phosphorylation/dephosphorylation directly regulates pp34 function.

THE *cdc2*⁺ gene encodes a 34K phosphoprotein (that is, of relative molecular mass 34,000) with protein-serine/threonine kinase activity, termed pp34, which plays a key part in regulating the eukaryotic cell cycle (reviewed in ref. 1). In the fission yeast, *Schizosaccharomyces pombe*, *cdc2*⁺ acts at two points in the cell cycle: first at 'start' where it is required for entry into S-phase and then again in late G2 where it regulates entry into mitosis². The homologue of *cdc2*⁺ in *Saccharomyces cerevisiae*, *CDC28*, is required at 'start'³ and possibly later in the cell cycle for mitosis⁴. A functionally equivalent and structurally similar gene has also been identified in human cells^{5,6} and other organisms^{7,8}. In frog^{9,10} and starfish¹¹, pp34 is a component of maturation promoting factor, which regulates onset of meiotic and mitotic M-phase during oocyte maturation and early embryogenesis. These observations have led to the proposal that pp34 is part of a universal control mechanism regulating onset of M-phase in all eukaryotic cells¹.

Entry into M-phase is believed to be brought about by activation of pp34 protein kinase activity, which rises to a peak at M-phase in all organisms investigated¹. In *S. pombe*, activation of pp34 requires a 56K cyclin-like protein¹² encoded by *cdc13*⁺ (refs 13–16) and the *cdc25*⁺ gene product^{12,17,18}. Cyclins have been found physically associated with pp34 in eukaryotic cells^{19–21}, and there is increasing evidence that *cdc25*⁺-like genes function in mitotic control in other organisms^{22,23}. Activation of pp34 in *S. pombe* is inhibited by the *wee1*⁺ gene product, a putative protein-serine/threonine kinase²⁴. In starfish¹¹, frog^{25,26}, and mammalian cells (refs 27, 28; C. Norbury, personal communication), protein kinase activation at M-phase is associated with pp34 dephosphorylation. Anti-phosphotyrosine antibodies have been used to demonstrate that in frog²⁵ and mammalian cells²⁸ dephosphorylation occurs on tyrosine and this has been confirmed in mammalian cells by phosphoamino-acid analysis (ref. 28; C. Norbury, personal communication). A role for tyrosine phosphorylation in regulating pp34 is of particular interest, given the importance of tyrosine-protein kinases

in the control of mammalian cell growth. However, in both *S. pombe*²⁹ and *S. cerevisiae*³⁰, pp34 phosphorylation levels have been reported not to change during the cell cycle and phosphoamino-acid analyses have failed to detect phosphotyrosine in the protein^{31,32}. Indeed, it is not even clear if tyrosine phosphorylation of any protein occurs in unicellular eukaryotes such as yeast, although low levels of phosphotyrosine³³ and protein-tyrosine kinase activity³⁴ in *S. cerevisiae* have been reported.

In this paper we establish that *S. pombe* pp34 is phosphorylated on tyrosine as well as on serine and threonine, the first demonstration of phosphotyrosine in this unicellular organism. Using highly synchronized cultures, we show that dephosphorylation of tyrosine and threonine residues occurs as *S. pombe* cells enter mitosis. We have localized the single detectable site of tyrosine phosphorylation in pp34 to Tyr 15, a residue within the proposed ATP-binding domain. Replacement of this tyrosine with phenylalanine abolishes tyrosine phosphorylation of pp34 and leads to premature advancement of cells into mitosis. This demonstrates that tyrosine phosphorylation/dephosphorylation of pp34 directly regulates entry of cells into mitosis.

Dephosphorylation during mitosis

To examine the phosphorylation state of pp34, we used an antiserum, hereafter referred to as PN24, raised against a peptide corresponding to the C-terminal seven amino acids of the *cdc2*⁺ gene product²⁹. Immunoprecipitation from ³²P-labelled wild-type *S. pombe* (strain 972) with PN24 revealed a major 34K band following electrophoresis and autoradiography (Fig. 1a, lane 1). The specificity of this reaction was demonstrated by the ability of the C-terminal peptide to block immunoprecipitation of pp34 when pre-incubated with the antiserum (Fig. 1a, lane 2). Also, one-dimensional peptide mapping of the immunoprecipitated 34K protein in comparison with bacterially produced pp34 using the chemical cleavage reagent, *N*-chlorosuccinimide⁵ established that the immunoprecipitated protein was the product of the *cdc2*⁺ gene (data not shown).

The relative level of pp34 phosphorylation at various points during the cell cycle was examined by immunoprecipitating pp34 from ³²P-labelled temperature-sensitive *cdc* strains arrested at their nonpermissive temperatures. Specifically, we used the *cdc25-22* and *cdc13-117* strains to block cells either in late G2 or in mid-mitosis, respectively. There was four times more ³²P in pp34 isolated from cells blocked in late G2 than there was in pp34 immunoprecipitated from cells arrested in mitosis (Fig. 1b, lanes 1 and 2). When isolated from exponentially growing cells, pp34 contained an intermediate level of ³²P (data not shown). Consistent with the lower ³²P content of pp34 in mitotically arrested cells, a decrease in pp34 phosphorylation was observed as cells entered mitosis when released from a late G2 block (Fig. 1c).

Tyrosine phosphorylation

Immunoprecipitates of pp34 from various ³²P-labelled cells were partially hydrolysed with acid to determine their phosphoamino-acid composition. All three phosphoamino acids were found in pp34 from exponentially growing cells (Fig. 2a). Phosphothreonine was most prominent, phosphotyrosine somewhat less

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abundant, and phosphoserine was barely detectable. The phosphoamino-acid content of pp34 from cells blocked in late G2 was slightly different in that phosphoserine was not detectable even in very long exposures and the level of phosphotyrosine was roughly equivalent to the level of phosphothreonine (Fig. 2b). The ratio of phosphothreonine to phosphotyrosine in pp34 was nearly 1:1 in cells blocked in mitosis (Fig. 2c) or released from a G2 block for 30 min (Fig. 2d), when overall phosphorylation is reduced. This indicates that dephosphorylation of both threonine and tyrosine residues occurs as cells traverse the boundary between G2 and M-phase.

The phosphotyrosine content of pp34 as cells entered mitosis was also examined with anti-phosphotyrosine antibodies. We immunoprecipitated pp34 from unlabelled *cdc25-22* cells arrested in late G2 at 36 °C and released for 30, 45 and 60 min

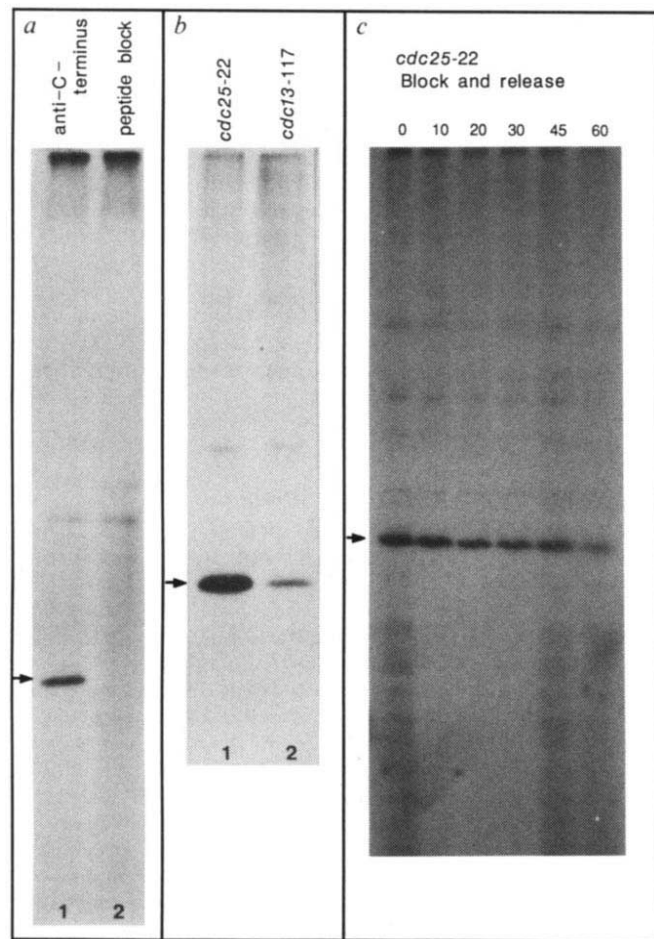


FIG. 1 Phosphorylation of pp34 during the G2-M phase transition. *a*, Wild-type *S. pombe* strain 972 was labelled with [32 P]orthophosphate and lysed as described⁴⁴. The pp34 protein was immunoprecipitated with excess antibody raised against the C-terminal seven amino acids of pp34 (ref. 29) (lane 1) or with the same antibody pre-incubated with 100 ng of the C-terminal peptide (lane 2). *b*, Immunoprecipitates of pp34 from equivalent amounts of 32 P-labelled *cdc25-22* (lane 1) or *cdc13-117* (lane 2) cells arrested at 36 °C. *c*, Immunoprecipitates of pp34 from equal numbers of *cdc25-22* cells labelled with [32 P]orthophosphate for 4.5 h at 36 °C and shifted to 25 °C for 0, 10, 20, 30, 45 and 60 min. Arrows indicate the position of pp34.

METHODS. *S. pombe* cells (5 or 10 ml) at $2-4 \times 10^6$ cells per ml were labelled with 1–3 mCi [32 P]orthophosphate in reduced phosphate minimal medium as described earlier^{29,44}. Wild-type strain 972 was labelled at 30 °C for 4 h and *cdc25-22* and *cdc13-117* strains at 36 °C for 4 or 4.5 h. Exponentially growing unlabelled wild-type cells (10^8) were added and lysis was performed as reported²⁹ with modifications⁴⁴. The pp34 protein was visualized by autoradiography after separation on SDS-17.5% polyacrylamide gels. Exposure was for 3–16 h at –70 °C with intensifying screens. Gel bands were quantitated by scintillation counting.

by shifting to the permissive temperature of 25 °C. The amount of phosphotyrosine in pp34 decreased 4–5-fold within 30–45 min of release, whereas the level of protein did not change appreciably (Fig. 3a, b). This decrease in reactivity with anti-phosphotyrosine antibodies occurred with the same kinetics as the decrease in overall 32 P content of pp34 observed in [32 P]-orthophosphate labelling experiments (compare Fig. 1c with Fig. 3a).

Tyrosine phosphorylation site

As a first step towards identifying the site(s) of tyrosine phosphorylation, we examined the phosphopeptide composition of pp34. The patterns of pp34 tryptic phosphopeptides were qualitatively identical when derived from exponentially growing wild-type *S. pombe* cells (Fig. 4a) or from cells arrested in late G2 (Fig. 4b). The pp34 protein contained one major phosphopeptide, termed phosphopeptide 1, and a cluster of minor peptides, collectively termed phosphopeptide 2 (Fig. 4a, b). Phosphopeptide 1 contained only phosphotyrosine (Fig. 4c) and was the only peptide to contain detectable levels of phosphotyrosine. Phosphopeptide 2 contained phosphothreonine (Fig. 4d). The low yield of the phosphothreonine-containing peptide relative to the phosphotyrosine-containing peptide is probably due to the relative insolubility of phosphopeptide 2 in our buffer system, a characteristic of large hydrophobic peptides. We were unable to detect a phosphoserine-containing tryptic phosphopeptide, presumably because of insufficient 32 P-labelled sample.

We were able to localize the tryptic peptide-containing phosphotyrosine to the N-terminal 3–4K fragment of the protein based on the following observations. First, *N*-chlorosuccinimide cleaves pp34 to generate a characteristic pattern of partial cleavage products ranging from ~18–32K (ref. 5). All of these fragments contain the N-terminus of the protein (ref. 5; unpublished observations). Because we found that all of the 32 P-labelled pp34 *N*-chlorosuccinimide fragments contained phosphotyrosine (data not shown), the tyrosine phosphorylation site had to lie within the 18K N-terminal fragment of the protein. Second, the largest fragment (~28K) derived from pp34 by partial *S. aureus* V8 protease digestion, fragment A (Fig. 4e), lacked phosphotyrosine (Fig. 4g). Because fragment A is generated by removal of a section of ~4K from the N-terminus of the protein (data not shown), the site of tyrosine phosphorylation was most probably in this ~4K N-terminal piece of pp34.

There are three tyrosine residues in this region of the protein which lie in two tryptic peptides³⁵. One tryptic peptide (amino acids 1–6) contains Tyr 4 and the other (amino acids 10–20) contains both Tyr 15 and Tyr 19. The tryptic peptide containing Tyr 4 was unlikely to be phosphopeptide 1 for two reasons. First, its theoretical mobility in our two-dimensional peptide mapping system did not correspond to the migration of phosphopeptide 1. Second, phosphopeptide 1 comigrated in our two-dimensional separations with a phosphotyrosine-containing tryptic peptide derived from murine pp34, strongly suggesting that a peptide with the identical sequence is phosphorylated in murine pp34 (C. Norbury, K.L.G. and P.N., unpublished observations). Comparison of the murine⁷ and *S. pombe* pp34 sequences³⁵ revealed that the only tryptic peptide in the relevant N-terminal region of pp34 present in both murine and *S. pombe* pp34 was the peptide containing Tyr 15 and Tyr 19.

To establish that phosphopeptide 1 contained amino acids 10–20, we synthesized this 32 P-labelled tryptic peptide *in vitro* and compared its migration in two-dimensional separations with that of phosphopeptide 1. To generate this 32 P-labelled tryptic peptide *in vitro*, a peptide corresponding to amino acids 7–26 of pp34 was synthesized (Fig. 5a), phosphorylated by immunoprecipitates of pp60^{v-src} (Fig. 5b) on tyrosine (data not shown), and cleaved with trypsin. The resultant synthetic tryptic phosphopeptide (Fig. 5c, panel A) comigrated with phosphopeptide 1 (Fig. 5c, panel C). These data indicated that

phosphopeptide 1 was a phosphotyrosine-containing peptide corresponding to amino acids 10–20.

From the migration of phosphopeptide 1 at different pHs, it appeared to contain only one phosphate moiety (data not shown) indicating that Tyr 15 and Tyr 19 were not phosphorylated in the same peptide molecule. To rule out the possibility that phosphopeptide 1 consisted of a mixture of two phosphopeptides, one phosphorylated at Tyr 15 and the other at Tyr 19, phosphopeptide 1 was cleaved with thermolysin. As shown in Fig. 5a, there are two potential thermolytic cleavage sites between Tyr 15 and Tyr 19. If both tyrosine residues were phosphorylated, cleavage with thermolysin would generate at least two distinct phosphopeptides. However, as phosphopeptide 1 yielded only one phosphopeptide on thermolytic cleavage (Fig. 5c, panels D and E), then only one tyrosine residue could be the site of phosphorylation *in vivo*.

Tyrosine substitutions

The codons for Tyr 15 and Tyr 19 were changed independently by site-directed mutagenesis to encode the structurally similar amino acid phenylalanine. The wild-type and mutant proteins were expressed from multicopy plasmids in a strain in which the *cdc2* gene had been deleted. These cells were labelled with [³²P]orthophosphate and the phosphoamino-acid composition of the *cdc2* proteins was examined. Although the *cdc2*⁺ and *cdc2.Phe19* proteins contained all three phosphoamino acids (Fig. 6a, panels A and C), phosphotyrosine was absent in the *cdc2.Phe15* protein (Fig. 6a, panel B). This result confirmed that Tyr 15 was the site of phosphorylation.

The phenotypes of cells deleted for *cdc2*⁺ and expressing plasmid-borne *cdc2*⁺ or *cdc2.Phe19* were indistinguishable from that of wild-type cells (Fig. 6b, panels A and B). In contrast, cells expressing *cdc2.Phe15* grew very slowly and had the general appearance of 'wee' mutants^{36,37}, septating at an average length of 7 μ m rather than at the wild-type size of 14 μ m (Fig. 6b, panels C–F). An abnormally high percentage of the cells contained septa (some very wide) and a large proportion of the

cells were either anucleate, binucleate, had nuclei that were cut by septa, or had fragmented nuclei (Fig. 6b, panels C–F). These pleiotropic and deleterious effects of the *cdc2.Phe15* mutant are not observed in other single *wee* mutants such as *wee1-50*, *cdc2-1w*, or *cdc2-3w* (refs 36, 37). However, they are similar to the effects generated by the double mutant, *wee1-50 cdc2-3w* (ref. 24). This strain undergoes mitotic catastrophe at the *wee1-50* restrictive temperature; the cells enter mitosis so early that

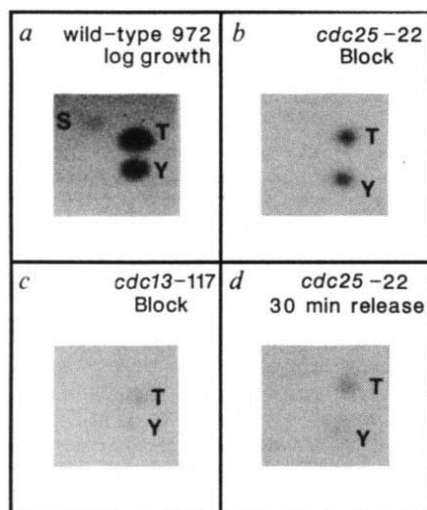


FIG. 2 Phosphoamino-acid compositions of pp34: a, pp34 from exponentially growing wild-type cells; b, pp34 from *cdc25-22* cells labelled at 36 °C for 4.5 h; c, pp34 from *cdc25-22* cells labelled at 36 °C and shifted to 25 °C for 30 min; d, pp34 from *cdc13-117* cells labelled at 36 °C. Key: S, phosphoserine; T, phosphothreonine; Y, phosphotyrosine.

METHODS. ³²P-labelled pp34 immunoprecipitates were obtained as described in the legend to Fig. 1, subjected to partial acid hydrolysis, and the phosphoamino acids were resolved by two-dimensional thin-layer electrophoresis as described^{45,46}. The sample of wild-type pp34 was derived from twice the number of cells labelled with more [³²P]orthophosphate. Samples containing 50–350 c.p.m. were loaded onto thin-layer cellulose and exposed for 6 days at –70 °C with intensifying screens. Thin-layer electrophoresis was performed on a CBS Scientific apparatus.

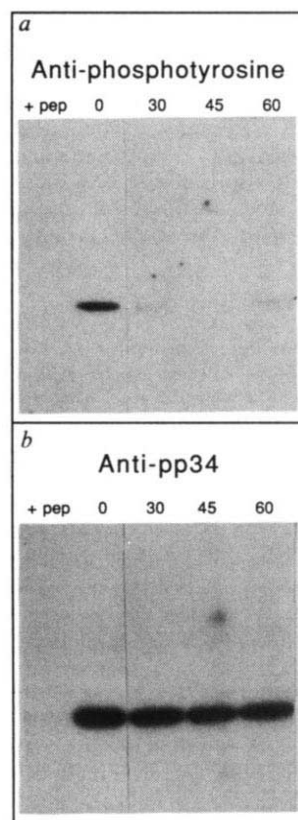


FIG. 3 Western blots of pp34 immunoprecipitates. Exponentially growing *cdc25-22* cells at 3×10^6 cells per ml were arrested at 36 °C for 4.5 h. At each time interval (0, 30, 45 and 60 min) after shift to 25 °C, 10^8 cells were lysed and subjected to immunoprecipitation with the anti-C-terminal peptide serum or at time 0 with the anti-C-terminal peptide serum pre-incubated with excess peptide as described in the legend to Fig. 1. Each immunoprecipitate was divided in half, and each half was resolved separately on SDS-17.5% polyacrylamide gels, blotted onto Immobilon-P, and probed with either anti-phosphotyrosine antibodies (a) or anti-pp34 antibodies (b), followed by ¹²⁵I-labelled protein A. The main band corresponds to pp34.

METHODS. The 3.4-kilobase *Pst*I genomic fragment of *cdc2*⁺ was cloned into the vector pTZ19R (Pharmacia). The four introns in *cdc2*⁺ were removed and a *Nde*I site was created at the initiating methionine by site-directed mutagenesis as described^{5,47}. A *Bam*HI site was introduced 36 base pairs downstream of the stop codon by changing the sequence CTATCC to GGATCC by site-directed mutagenesis. All site-directed mutagenesis was carried out with an Amersham *in vitro* mutagenesis kit according to the manufacturer's instructions. The *Nde*I–*Bam*HI *cdc2*⁺ fragment was subcloned into the bacterial expression vector pRK171 and expressed in the bacterial strain BL21(DE3) as reported previously^{5,31}. After lysis of the bacteria with a French press, *cdc2* protein was purified from insoluble inclusion bodies by solubilization in gel sample buffer and electroelution from SDS-polyacrylamide gels. Polyclonal rabbit serum was raised in rabbits by injection of 500 μ g of pp34 near the popliteal lymph node first in complete Freund's adjuvant and thereafter every four weeks with the same amount of protein mixed with incomplete Freund's adjuvant. One antiserum (4711) was used at a 1:500 dilution for western blotting. Western blotting with anti-pp34 antibodies and anti-phosphotyrosine antibodies (from M. Kamps and B. Sefton) was performed as described⁴⁸, except that 10 μ Ci of ¹²⁵I-labelled protein A at 2–10 μ Ci per μ g protein A was used to detect rabbit immunoglobulins. Exposures were for 10 days for a and 3 days for b at –70 °C with intensifying screens.

FIG. 4 Tryptic peptide and partial *S. aureus* V8 peptide analysis of pp34. *a*, Tryptic phosphopeptides of pp34 from ^{32}P -labelled exponentially growing 972 cells. *b*, Tryptic phosphopeptides of pp34 from ^{32}P -labelled *cdc25-22* cells arrested at 36 °C. Electrophoresis was at pH 4.72 in the horizontal dimension, with the anode on the left. The origins are indicated with arrowheads. *c*, Phosphoamino-acid analysis of phosphopeptide 1. *d*, Phosphoamino-acid analysis of phosphopeptide 2. *e*, One-dimensional peptide map of pp34 from *cdc25-22* cells arrested at 36 °C treated with 0 or 100 ng *S. aureus* V8 protease. *f*, Phosphoamino-acid analysis of undigested pp34. *g*, Phosphoamino-acid analysis of pp34 V8 fragment A.

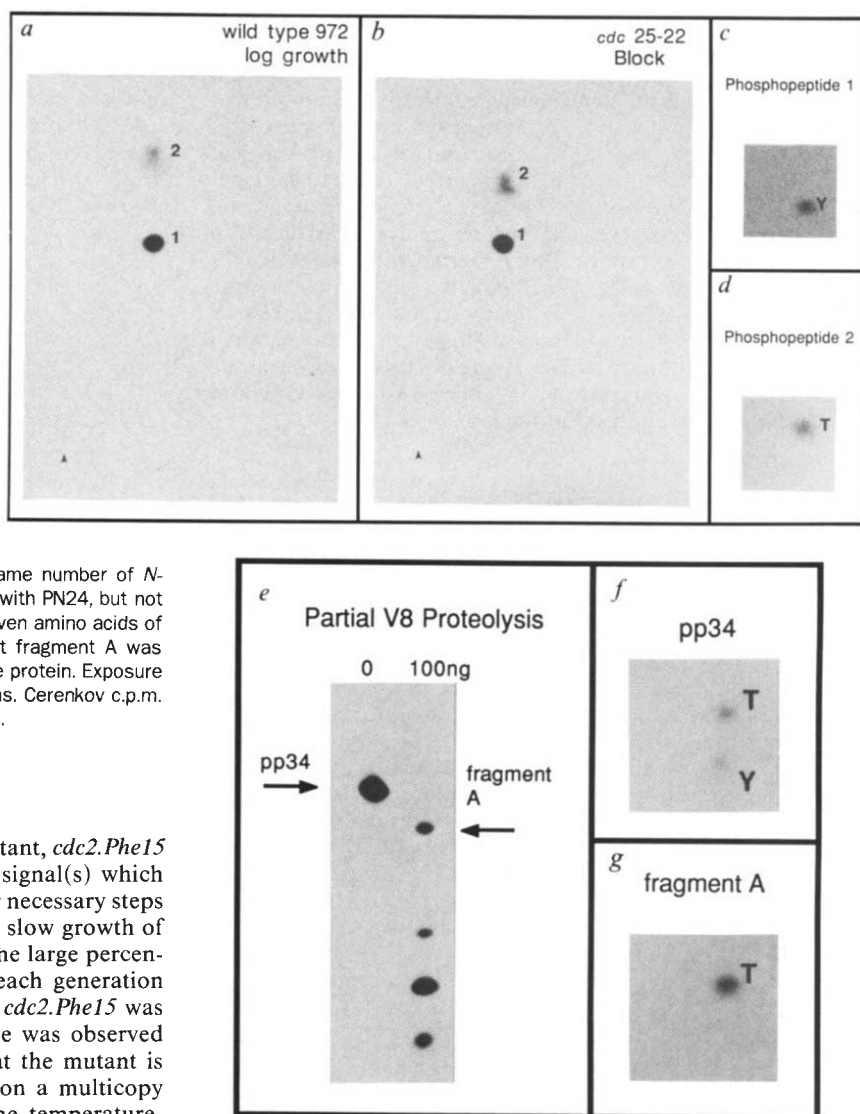
METHODS. ^{32}P -labelled pp34 immunoprecipitates were subjected to tryptic digestion, and the tryptic phosphopeptides were separated in two dimensions as detailed previously^{46,49}. Phosphoamino-acid analysis was as described in the legend to Fig. 2. One-dimensional peptide maps of pp34 were prepared on SDS-20% polyacrylamide gels with 0 or 100 ng of *S. aureus* V8 protease (Boehringer) as described⁴⁹. Fragment A yielded the same number of *N*-chlorosuccinimide products as intact pp34 and reacted with PN24, but not an antipeptide serum directed against the N-terminal seven amino acids of the protein²⁹ (data not shown). This demonstrated that fragment A was generated by cleavage of an N-terminal peptide from the protein. Exposure times were 4–6 days at –70 °C with intensifying screens. Cerenkov c.p.m. loaded onto thin-layer plates ranged from 50–350 c.p.m.

cell death frequently results. Like this double mutant, *cdc2.Phe15* appears to be independent of some inhibitory signal(s) which normally delays entry into mitosis until all other necessary steps in the cell cycle have been completed. The very slow growth of the *cdc2.Phe15* strain can be accounted for by the large percentage of inviable cells which are produced in each generation from lethal premature entry into mitosis. When *cdc2.Phe15* was expressed in *cdc2*⁺ strains, the same phenotype was observed (Fig. 6*b*, panels *G* and *H*), demonstrating that the mutant is dominant over wild-type *cdc2*⁺ when present on a multicopy plasmid. Also, *cdc2.Phe15* is able to rescue the temperature-sensitive mutant *cdc2-33*, providing further evidence that it encodes a functional *cdc2* protein. In addition, like the *wee* mutant *cdc2-3w*, it is able to rescue the conditionally lethal *cdc25-22* strain.

Discussion

We have shown in this paper that *S. pombe* pp34 is multiply phosphorylated in exponentially growing cells. It is maximally phosphorylated in late G2 and as cells enter mitosis, the level of pp34 phosphorylation decreases. The protein kinase activity of pp34 in *S. pombe* was shown in earlier reports^{12,17} to increase from a low level in late G2 (at a *cdc25-22* block point) to its maximum level in mitosis (during a *cdc25-22* release) with the same kinetics as described here for dephosphorylation. A decrease in pp34 phosphorylation as cells enter mitosis has been observed previously in starfish¹¹, frog^{25,26}, and mammalian^{27,28} cells. Because dephosphorylation in these higher eukaryotes is coincident with an increase in pp34 protein kinase activity towards histone H1, it has been proposed that dephosphorylation might regulate the activation of pp34 (refs 11, 25, 26, 28). The fact that pp34 enzymatic activity is inversely correlated with its level of phosphorylation during the G2–M transition in *S. pombe* and in several higher eukaryotes indicates that this potential regulatory mechanism has been highly conserved throughout eukaryotic evolution.

A previous investigation in our laboratory did not detect any changes in pp34 phosphorylation levels as *S. pombe* cells moved through the cell cycle²⁹. The most likely explanation for



the apparent discrepancy between our two reports lies in the methods used for obtaining synchronous cell cultures. In the present study, we used the temperature-sensitive mutations *cdc25-22* and *cdc13-117*, which arrest either in late G2 or mitosis, respectively. On shift from the restrictive to the permissive temperature, *cdc25-22* cells enter mitosis in a highly synchronous fashion. In the earlier study, cells were fractionated by size using an elutriation rotor²⁹. Although this method causes the least physiological disturbance to the cells, it does not achieve such good cell-cycle synchrony. If pp34 dephosphorylation in *S. pombe* is quite transient and cells arrived at a point in mitosis when dephosphorylation occurs somewhat asynchronously in our experiments, we would only expect to see a small overall change. Because we observed only a fourfold dephosphorylation of pp34 in cultures synchronized by a *cdc25-22* block and release, it is likely that dephosphorylation would have been extremely difficult to detect in cultures synchronized by elutriation. A small change in overall pp34 phosphorylation might also indicate that only a subpopulation of pp34 molecules in *S. pombe* is subject to dephosphorylation during the G2–M transition. Like the previous study on pp34 phosphorylation in *S. pombe*²⁹, phosphorylation changes were not detected in the *S. cerevisiae* homologue of pp34 during the cell cycle³⁰. More recently, however, a transient phosphorylation of the *CDC28* gene product on tyrosine residues has been observed (*S. Reed, personal communication*).

We have found both by phosphoamino-acid analysis and by

immunoblotting with anti-phosphotyrosine antibodies that pp34 contains phosphotyrosine. The phosphotyrosine content of pp34 is maximal in late G2 and decreases with the same kinetics as total ^{32}P content of the protein when cells enter mitosis. Phosphotyrosine was not detected in a previous study of pp34 phosphorylation, although phosphothreonine and phosphoserine were detected in roughly the same proportion that we have observed³¹. We do not know the reason for this discrepancy but suspect that it is due to the very different labelling conditions and cell densities used.

The *cdc2*⁺ protein kinase is the first yeast protein known to contain phosphotyrosine. There has been a report of an unknown protein reacting with anti-phosphotyrosine antibodies in yeast lysates but this has not been substantiated by phosphoamino-acid analysis³⁸. Because anti-phosphotyrosine antibodies have been reported to react in some circumstances with phosphohistidine, phosphothreonine and phosphoserine residues within proteins (ref. 39; R. Lindberg and T. Hunter, personal communication), reports relying solely on reactivity with anti-phosphotyrosine antibodies have been regarded with some scepticism.

Very small amounts of phosphotyrosine have been obtained from partial acid hydrolysates of total cell protein³³. However, other modifications of tyrosine such as adenylation can yield phosphotyrosine upon partial acid hydrolysis⁴⁰. Thus, whether the tiny amounts of phosphotyrosine detected in acid hydrolysates are actually due to modification of tyrosine residues by enzymatic phosphorylation remains an open issue. Tyrosine-protein kinase activity in yeast, on the other hand, has been demonstrated more definitively. A very low level of activity that phosphorylates a copolymer of glutamic acid and tyrosine on tyrosine has been identified in crude lysates of *S. cerevisiae*³⁴. In multicellular eukaryotes, tyrosine phosphorylation has been more readily detected and is intrinsic to more than 30 proteins⁴¹. Of the diverse array of protein-tyrosine kinases known, most appear to be associated with the plasma membrane, either as growth factor receptors or as putative transducers of extracellular stimuli⁴¹. Because such a highly elaborate system for responding to growth factors and other intercellular signals is unlikely to be required in a unicellular organism, it is probable that many fewer protein-tyrosine kinases exist in yeast. However,

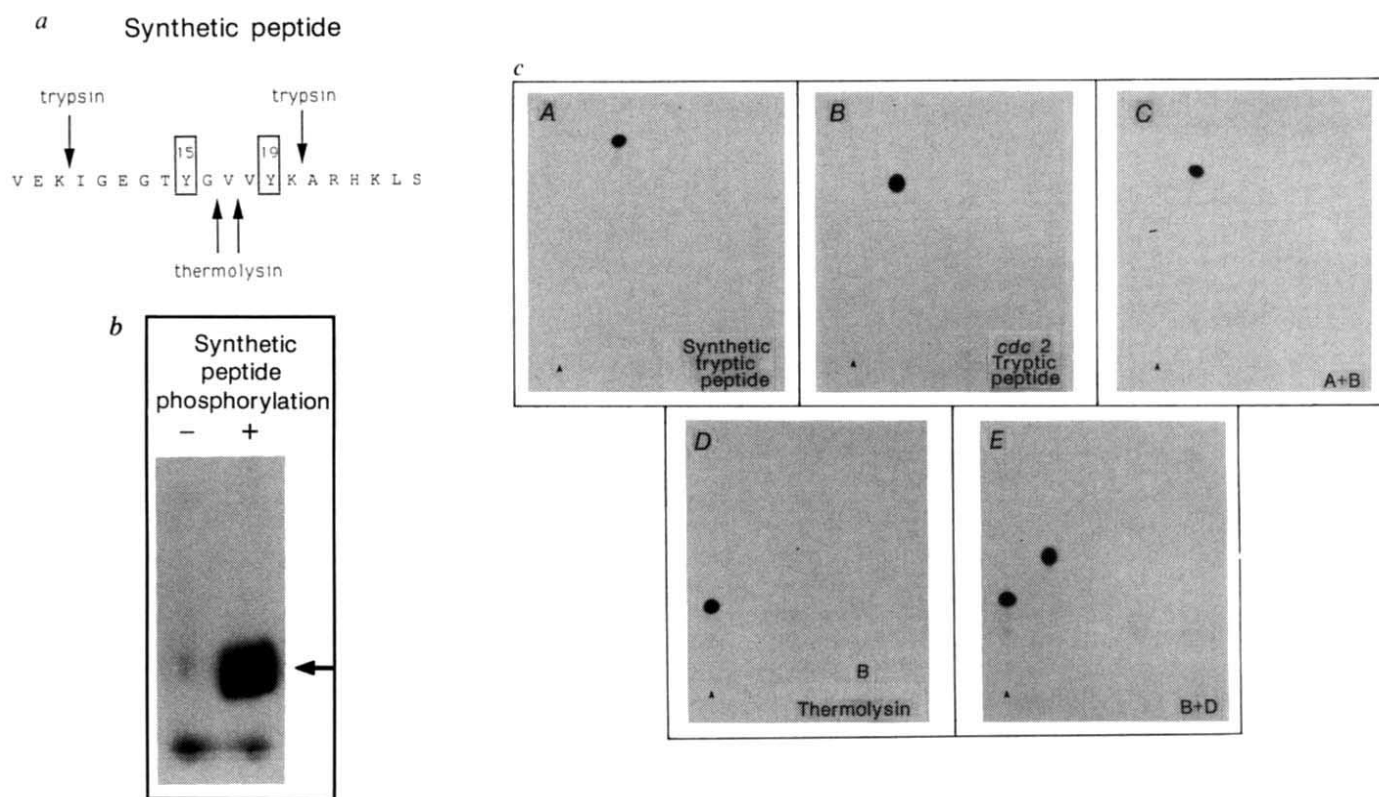


FIG. 5 Localization of the tyrosine phosphorylation site in pp34. *a*, Sequence of the synthetic peptide corresponding to amino acids 7–26 of pp34. Arrows indicate cleavage sites for trypsin and thermolysin. The two tyrosines are boxed. *b*, Phosphorylation of the synthetic peptide by pp60^{v-src} in the presence of [γ - ^{32}P]ATP. The plus and minus signs refer to reactions which did or did not contain the peptide, respectively. The arrow indicates the position of phosphorylated peptide. *c*, The synthetic peptide phosphorylated by pp60^{v-src} was cleaved with trypsin and resolved in two dimensions (panel A). The tryptic phosphopeptide containing phosphotyrosine shown in Fig. 4, *a* and *b*, was re-run separately (panel B). Equal Cerenkov c.p.m. of each tryptic phosphopeptide shown separately in panels A and B were mixed in panel C. The tryptic phosphopeptide shown in B was cleaved with thermolysin and resolved in two dimensions (panel D). Equal Cerenkov c.p.m. of each phosphopeptide run separately in panels B and D were mixed in panel E. Cerenkov c.p.m. loaded onto cellulose plates ranged from 60–100 c.p.m. Exposure times were 5 days at -70°C with intensifying screens. Electrophoresis was at pH 1.9 in the horizontal dimension with the anode on the left.

METHODS. A peptide corresponding to amino acids 7–26 of pp34 was synthesized using a Applied Biosystems 430A synthesizer and purified by

reverse-phase HPLC. Amino-acid analysis confirmed the authenticity of the sequence. Immunoprecipitation of pp60^{v-src} was from LA29-transformed rat-1 cells with monoclonal antibody 327 (ref. 50; provided by T. Hunter) as described previously⁴⁹. Immunoprecipitates of pp60^{v-src} were resuspended in 30 μl 20 mM Tris-HCl, pH 7.5, 10 mM MnCl_2 and 3 μl 100 mM Tris-HCl, pH 7.5, with or without 3 μg of the synthetic peptide. Reactions were initiated by the addition of 30 μCi [γ - ^{32}P]ATP and allowed to proceed at room temperature for 15 min. Immune complexes were pelleted by brief centrifugation and 10 μl of the supernatants were added to 10 μl of 2 $\times$ sample buffer. After heating to 100°C for 2 min, this 20- μl portion was resolved on an SDS-25% polyacrylamide gel and the phosphorylated peptide was visualized by autoradiography. The remainder of the synthetic peptide phosphorylated by pp60^{v-src} was purified by cellulose thin-layer electrophoresis at pH 1.9. It was eluted from the cellulose with pH 1.9 buffer, lyophilized, resuspended in 20 μl 50 mM ammonium bicarbonate, pH 8.0, and digested with 2 μg trypsin for 2 h at 37°C . Phosphopeptide 1 (Fig. 4a, b) was eluted from cellulose plates and digested with thermolysin as described above, except that cleavage was performed at 55°C . All other manipulations of phosphopeptides, including two-dimensional separation, were carried out as described previously^{46,49}.

the protein-tyrosine kinases that are present in yeast might have fundamental roles in the assessment of growth and/or nutritional status and relate that information to progress through the mitotic cell cycle.

There is a single detectable site of tyrosine phosphorylation in fission yeast pp34 and we have identified it as Tyr 15. This residue lies directly within a part of the postulated nucleotide binding domain of pp34, the GXGXXG motif⁴². The location of Tyr 15 leads us to suggest that the function of tyrosine phosphorylation in pp34 is to prevent ATP binding either sterically and/or by electrostatic repulsion, thereby inactivating the protein. This hypothesis is consistent with the observation that dephosphorylation of pp34 correlates with increased protein kinase activity. Modulation of nucleotide-binding ability by phosphorylation would represent a novel mechanism of protein kinase regulation. A comparison of protein kinase sequences⁴² reveals that *cdc2*⁺ and its functional homologues are not unique in containing a tyrosine at this position. It is possible, therefore, that other protein-serine/threonine and protein-tyrosine kinases might be regulated by tyrosine phosphorylation within their ATP-binding domains.

The potential role of tyrosine phosphorylation in the regulation of pp34 was addressed by changing the codon specifying Tyr 15 to that encoding phenylalanine and then examining the effect of this mutation in *S. pombe*. Based on the observation that *cdc2.Phe15* was able to rescue a temperature-sensitive allele

of *cdc2*, as well as a strain in which the *cdc2*⁺ gene had been disrupted (although the growth rate of the rescued strains was low), we conclude that the mutant *cdc2.Phe15* protein retains enzymatic activity. Cells expressing *cdc2.Phe15* either with or without *cdc2*⁺ entered mitosis at a small cell size and exhibited a wide range of abnormal phenotypes suggestive of mitotic catastrophe. Many were anucleate, binucleate, had septation defects, or had fragmented nuclei. Cell division in this strain resulted in many inviable progeny, accounting for its slow doubling time. The ability of this strain to grow at all indicates that this single mutant is not quite as detrimental as the double mutant *wee1-50 cdc2-3w*. As mentioned earlier, the combination of *wee1-50* and *cdc2-3w* alleles results in mitotic catastrophe preventing formation of colonies at the *wee1-50* nonpermissive temperature²⁴. Thus, although *cdc2.Phe15* appears to bypass much of the normal inhibition acting to time entry into mitosis properly, this mutant does not appear to be de-regulated completely.

The *cdc2.Phe15* mutant protein is still phosphorylated on threonine and serine residues. We have shown in this report that phosphothreonine as well as phosphotyrosine decreases as cells enter mitosis, suggesting that phosphothreonine might also be involved in the regulation of pp34 activity. It will be interesting to identify the sites of threonine and serine phosphorylation and assess the role of these phosphorylation events in the regulation of pp34.

Because pp34 is regulated by tyrosine phosphorylation and dephosphorylation, presumably at least one protein-tyrosine kinase and phosphatase contribute to regulation of the *S. pombe* mitotic cell cycle. The *cdc25* gene function, which normally is required for activation of pp34, is bypassed by *cdc2.Phe15* because *cdc2.Phe15* can rescue a temperature-sensitive mutation of this gene. Although the function of *cdc25* is unknown and its amino-acid sequence does not reveal similarities to the sequence of any published protein phosphatase or protein kinase inhibitor¹⁸, these findings would be consistent with a role for *cdc25* in the activation of a protein-tyrosine phosphatase or the inhibition of a protein-tyrosine kinase.

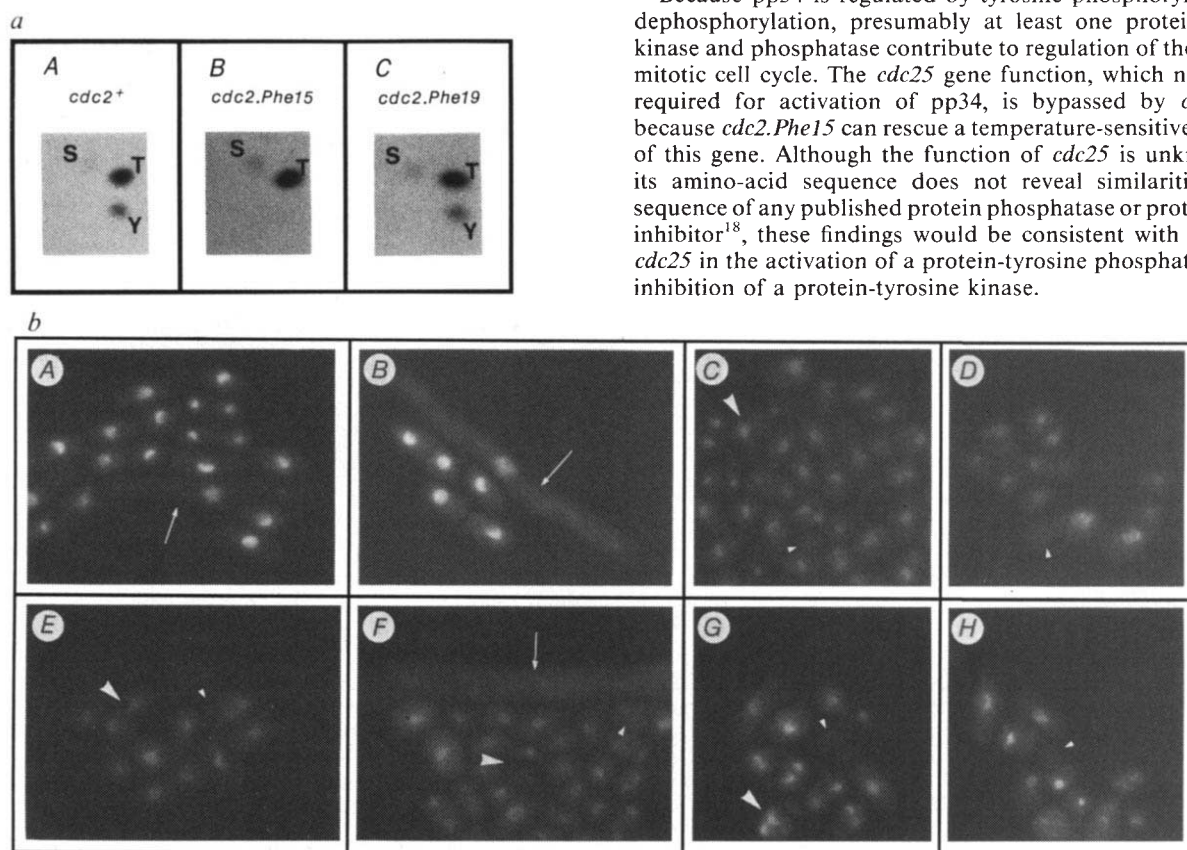


FIG. 6 Phenotype of *cdc2* mutants. *a*, Phosphoamino-acid analyses of pp34 proteins from *S. pombe* cells with a deleted *cdc2*⁺ gene complemented by plasmid-borne *cdc2*⁺, *cdc2.Phe15*, or *cdc2.Phe19* labelled with [³²P]-orthophosphate. *b*, DAPI staining. *S. pombe* cells with a deleted *cdc2*⁺ gene complemented by plasmid-borne (A) *cdc2*⁺, (B) *cdc2.Phe15*, or (C, D, E and F) *cdc2.Phe15*. *S. pombe* *leu1-32* cells complemented by a plasmid containing *LEU2* and *cdc2.Phe15* (G and H). Long arrows indicate cells which have lost *cdc2*-containing plasmids and are *cdc*⁻. Arrowheads point to cells undergoing abnormal septation and anucleate cells. METHODS. Mutagenesis of Tyr 15 and Tyr 19 in *cdc2*⁺ was carried out as described in the legend to Fig. 3 with 20-base-long mutagenic oligo-

nucleotides. The mutant genes were transformed into the diploid strain, *h*⁺/*h*⁹⁰, *leu1-32/leu1-32*, *ura 4-D18/ura 4-D18*, *cdc2*⁺/*cdc2:ura4*⁺, *ade6-704/ade6-704*, on a multicopy plasmid containing *LEU2* (ref. 44). *Leu*⁺ transformants were allowed to sporulate and unsporulated cells were digested with helicase as described⁴⁴. Spores were plated onto minimal plates lacking leucine and uracil. Colonies obtained from this selection were transferred to complete YEA medium⁴⁴. The same vector was also used to transform *leu1-32* cells with *cdc2.Phe15*, and *leu*⁺ transformants were selected. ³²P-labelling, immunoprecipitation and phosphoamino-acid analysis were performed as described in the legends to Figs 1 and 2. DAPI staining was performed as reported elsewhere⁴⁴.

The tyrosine residue that is phosphorylated in pp34 is conserved among all known pp34 homologues from yeast to humans^{6-8,32,35} and it is now known that pp34 contains phosphotyrosine in human⁴³, mouse²⁸, frog²⁵, *S. pombe* (this report) and *S. cerevisiae* (S. Reed, personal communication) cells. It is possible, therefore, that the tyrosine residue phosphorylated in *S. pombe* pp34 is also phosphorylated in pp34 from other species and that control of pp34 activity by dephosphorylation of this residue might be conserved throughout evolution. Purified mammalian pp34 can be phosphorylated on tyrosine by pp60^{v-src}

*in vitro*⁴³, as can a synthetic peptide corresponding to amino acids 7-26 of *S. pombe* pp34. However, it should be kept in mind that pp60^{v-src} is capable of phosphorylating nonphysiologically relevant protein substrates *in vitro*⁴¹ and that there is no evidence as yet that pp60^{c-src} directly phosphorylates pp34 *in vivo* in any system. The identification and characterization of the putative protein-tyrosine kinase and phosphotyrosine phosphatase responsible for regulating pp34 phosphotyrosine levels in *S. pombe* should enhance our understanding of cell-cycle control in all eukaryotes. □

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1. Nurse, P. *Nature* (in the press).
2. Nurse, P. & Bissett, Y. *Nature* **292**, 558-560 (1981).
3. Reed, S. *Genetics* **95**, 561-577 (1980).
4. Piggett, J. R., Rai, R. & Carter, B. L. *A. Nature* **298**, 391-393 (1982).
5. Draetta, G., Brizuela, L., Potashkin, J. & Beach, D. *Cell* **50**, 319-325 (1987).
6. Lee, M. G. & Nurse, P. *Nature* **327**, 31-35 (1987).
7. Cisek, L. J. & Corden, J. L. *Nature* **339**, 679-684 (1989).
8. Krek, W. & Nigg, E. A. *EMBO J.* **8**, 3071-3078 (1989).
9. Dunphy, W. G., Brizuela, L., Beach, D. & Newport, J. *Cell* **54**, 423-431 (1988).
10. Gautier, J., Norbury, C., Lohka, M., Nurse, P. & Maller, J. *Cell* **54**, 433-439 (1988).
11. Labbe, J. C. *et al. Cell* **57**, 253-263 (1989).
12. Moreno, S., Hayles, J. & Nurse, P. *Cell* **58**, 361-372 (1989).
13. Booher, R. N. & Beach, D. *EMBO J.* **7**, 2321-2327 (1988).
14. Goebel, M. & Byers, B. *Cell* **54**, 739-740 (1988).
15. Hagan, I., Hayles, J. & Nurse, P. *J. Cell Sci.* **91**, 587-595 (1988).
16. Solomon, M., Booher, R., Kirschner, M. & Beach, D. *Cell* **54**, 738-739 (1988).
17. Booher, R. N., Alfa, C. E., Hyams, J. S. & Beach, D. *H. Cell* **58**, 485-497 (1989).
18. Russell, P. & Nurse, P. *Cell* **45**, 145-153 (1986).
19. Draetta, G. *et al. Cell* **56**, 829-838 (1989).
20. Meijer, L. *et al. EMBO J.* **8**, 2275-2282 (1989).
21. Pines, J. & Hunter, T. *Cell* **58**, 833-846 (1989).
22. Edgar, B. A. & O'Farrell, P. H. *Cell* **57**, 177-187 (1989).
23. Russell, P., Moreno, S. & Reed, S. I. *Cell* **57**, 295-303 (1989).
24. Russell, P. & Nurse, P. *Cell* **49**, 559-567 (1987).
25. Dunphy, W. G. & Newport, J. W. *Cell* **58**, 181-191 (1989).
26. Gautier, J., Matsukawa, T., Nurse, P. & Maller, J. *Nature* **339**, 626-629 (1989).
27. Draetta, G. & Beach, D. *Cell* **54**, 17-26 (1988).
28. Morla, A. O., Draetta, G., Beach, D. & Wang, J. Y. *J. Cell* **58**, 193-203 (1989).
29. Simanis, V. & Nurse, P. *Cell* **45**, 261-268 (1986).

30. Hadwiger, J. A. & Reed, S. I. *Molec. cell. Biol.* **8**, 2976-2979 (1988).
31. Potashkin, J. A. & Beach, D. *Curr. Genet.* **14**, 235-240 (1988).
32. Reed, S. I., Hadwiger, J. A. & Lorincz, A. T. *Proc. natn. Acad. Sci. U.S.A.* **82**, 4055-4059 (1985).
33. Castellanos, R. M. P. & Mazon, M. J. *J. biol. Chem.* **260**, 8240-8242 (1985).
34. Schieven, G., Thorne, J. & Martin, G. S. *Science* **231**, 390-393 (1986).
35. Hindley, J. & Phear, G. A. *Gene* **31**, 129-134 (1984).
36. Fantes, P. A. *J. Bact.* **146**, 746-754 (1981).
37. Nurse, P. & Thuriaux, P. *Genetics* **96**, 627-637 (1980).
38. Grandori, R., Vai, M., Di Renzo, M. F., Alberghina, L. & Popolo, L. *Biochem. biophys. Res. Commun.* **160**, 887-896 (1989).
39. Frackelton, A. R. Jr, Ross, A. H. & Eisen, H. N. *Molec. cell. Biol.* **3**, 1343-1352 (1983).
40. Shapiro, B. M. & Stadtman, E. R. *J. biol. Chem.* **243**, 3769-3771 (1968).
41. Hunter, T. & Cooper, J. A. *A. Rev. Biochem.* **54**, 897-930 (1985).
42. Hanks, S. K., Quinn, A. M. & Hunter, T. *Science* **241**, 42-52 (1988).
43. Draetta, G. *et al. Nature* **336**, 738-744 (1988).
44. Moreno, S., Nurse, P. & Klar, A. *Meth. Enzym.* (in the press).
45. Cooper, J. A., Sefton, B. M. & Hunter, T. *Meth. Enzym.* **99**, 387-402 (1983).
46. Hunter, T. & Sefton, B. M. *Proc. natn. Acad. Sci. U.S.A.* **77**, 1311-1315 (1980).
47. Booher, R. & Beach, D. *Molec. cell. Biol.* **6**, 3523-3550 (1986).
48. Kamps, M. P. & Sefton, B. M. *Oncogene* **2**, 305-315 (1988).
49. Gould, K. L. & Hunter, T. *Molec. cell. Biol.* **8**, 3345-3356 (1988).
50. Lipsich, L. A., Lewis, A. J. & Brugge, J. S. *J. Virol.* **48**, 352-360 (1983).

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Sequence of an unusually large protein implicated in regulation of myosin activity in *C. elegans*

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The *Caenorhabditis elegans* gene *unc-22* encodes a very large muscle protein, called twitchin, which consists of a protein kinase domain and several copies of two short motifs. The sequence of twitchin has unexpected similarities to the sequences of proteins of the immunoglobulin superfamily, cell adhesion molecules and vertebrate muscle proteins, including myosin light-chain kinase. These homologies, together with results from earlier genetic and molecular analyses, indicate that twitchin is involved in a novel mechanism of myosin regulation.

THE gene *unc-22* is one of more than 40 genes important for muscle assembly and function that have been identified in the nematode *Caenorhabditis elegans*¹. Mutant animals that lack the *unc-22*-encoded protein (Unc-22) have a unique and puzzling phenotype: a nearly constant twitching of the body muscles. These muscles are unable to develop or sustain normal contractions. Instead, small regions within the myofilament lattice of individual muscle cells contract transiently in the absence of contraction of the adjacent lattice. Mutations are readily recovered and most of these are conditionally dominant^{2,3}. All available extragenic suppressors of *unc-22* mutants are missense mutations of the main body wall myosin heavy-chain gene *unc-54*^{4,5}, indicating that the Unc-22 protein may act through myosin to regulate contraction. The *unc-22* gene has been cloned by transposon tagging⁶ and this has permitted a molecular analysis of the gene⁷.

The protein product of *unc-22*, which we call twitchin after the distinctive mutant phenotype, is located in the myosin-containing regions of muscle cells and has a relative molecular

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mass of ~600,000 (M_r 600 K)⁷. Several very large proteins of unknown function, such as titin⁸⁻¹³, nebulin¹⁴ and dystrophin¹⁵, have been found in vertebrate muscle, but their relationships to twitchin are unclear. To understand the possible role of twitchin in muscle and to facilitate the search for homologous genes in other organisms, we determined the sequence of the *unc-22* gene.

The protein encoded by *unc-22*

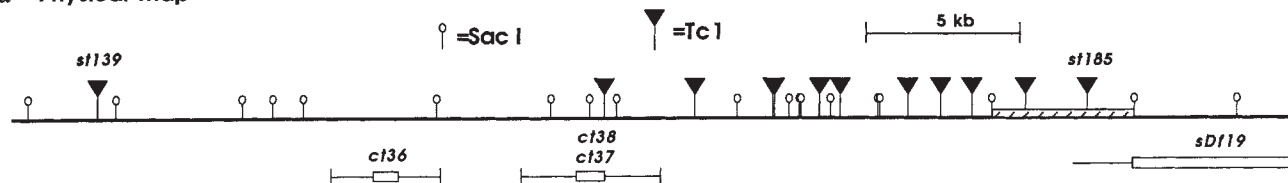
The *unc-22* gene had been estimated to span more than 30 kilobases (kb), as deduced from DNA insertions and deletions that affect *unc-22* activity⁷ (Fig. 1a). We sequenced a cosmid¹⁶ and two adjacent restriction fragments to cover the entire region (Fig. 1b). The cosmid was sequenced by using a shotgun library prepared from total cosmid DNA, and the restriction fragment sequences were determined by conventional means. The result-

ing 47,081 base pairs (bp) of continuous sequence (GenBank EMBL accession number X15423) included the sites¹⁷ of the two most extreme rearrangements (*st139* and *st185*; Fig. 1a), with 32,160 bp separating them.

The *unc-22* coding sequence in this genomic sequence was identified by using codon bias¹⁸ and the consensus donor and acceptor sites of *C. elegans* introns¹⁹. The repeated nature of the encoded protein sequence (see below) also aided in the exon-intron assignments. Furthermore, complementary DNA sequence (Fig. 1b) confirmed the four 3'-most exons and also identified the poly(A) site (at position 34,386).

The coding sequence is divided among 14 exons, the largest of which is 9,613 bp. The 13 introns have the base composition and size typical of *C. elegans* introns¹⁹. The ATG at position 14,070 has tentatively been assigned as the initiator methionine

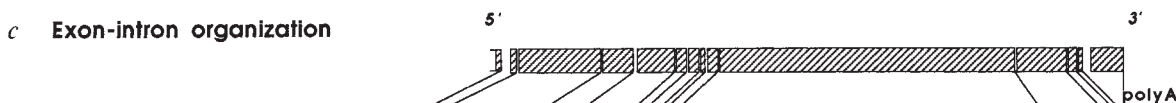
a Physical map



b DNA clones



c Exon-intron organization



d Protein domains

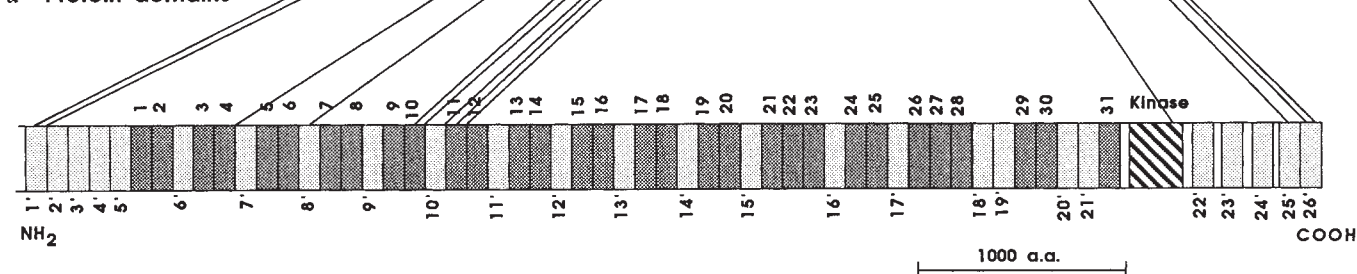


FIG. 1 The *unc-22* gene and its encoded polypeptide. **a**, Physical map of the *unc-22* region. The restriction map, the position of 12 transposon *Tc*I insertions that result in the *unc-22*-mutant phenotype, and the positions of the deficiencies *sDf19*, *ct36*, *ct37* and *ct38* are shown (modified from ref. 7). The position of the breakpoint of the deficiency *sDf19* orientates the physical map relative to the genetic map^{6,36-38}. The open boxes represent the extent of deletions and the lines extending from them indicate uncertainties of position. The stippled box to the right indicates the 4.6-kb fragment used to probe the cDNA libraries. **b**, DNA clones that were sequenced. A portion of the cosmid clone C32B3, and two small overlapping plasmid genomic clones pSL4 and pSL7 are shown. LSR1, W3-2 and W11b on the right are cDNA clones. **c**, Exon-intron organization of the *unc-22* gene. The uncertainty in the 5' end of the transcript is denoted by the open box at the left. The polyadenylation addition site is indicated at the right. The scale is the same as in **a** and **b** and the gene is aligned with the physical map in **a**. **d**, Schematic representation of domains in the twitchin amino-acid sequence. The single protein kinase domain is denoted by the hatched box, the 31 copies of motif I are represented as darkly shaded boxes (1'-31'), and the 26 copies of motif II are represented as lightly shaded boxes (1'-26'). Small regions lacking homology either to themselves or to motifs I or II are indicated by the unshaded areas. Uncertainty in the translation start site is indicated by the open box at the left.

METHODS. A single shotgun M13 library was constructed³⁹ from the entire cosmid C32B3 (ref. 16). Dideoxy sequencing^{40,41} reaction products from all clones were fractionated on polyacrylamide wedge-spacer gradient gels⁴² and from one-third of the clones, on non-gradient gels for extended readings. Sequences were compiled and analysed with the programs of R. Staden⁴³. After analysis of ~600 independent clones with ~1,050 gel readings, the sequence lay in 20 contigs. To close these gaps, thirty oligonucleotide primers (each 17-18 bases; synthesized by the Emory Microchemical Facility) were used to obtain a single contiguous sequence of 44,977 bp. To resolve ambiguities and read both strands of the cosmid insert we used 10 more oligonucleotide primers. The entire 47,081-bp sequence (including inserts of pSL4 and pSL7) was determined on both strands. Probable exons were determined by Staden's codon usage method, initially by using a codon table for abundantly expressed genes in *C. elegans*¹⁸, and later using a codon table compiled from the *unc-22* gene itself. Candidate introns were detected by searching for consensus donor and acceptor sequences, and for conformity to the typical *C. elegans* intron¹⁹. The deduced amino-acid sequence was compared with others in the Protein Identification Resource database (release 20) and all possible reading frames of each GenBank DNA sequence (release 59) using the HOMOLOG program of GENEPRO (Riverside Scientific Enterprises, Seattle, Washington).

the partial copy represents a complete copy of motif II and, indeed, yet another copy of motif II is upstream of that (N. Olson and A. R. Means, manuscript in preparation). By contrast, the rskMLCK lacks copies of either motifs I or II and its similarity to twitchin is limited to the protein kinase domain.

The high degree of similarity of twitchin with csmMLCK and rskMLCK in their kinase domains strongly indicates that one function of twitchin is to phosphorylate a muscle protein, presumably the regulatory myosin light chain. The extended similarity between twitchin and csmMLCK (37% identical and 47% similar allowing for conservative changes over a sequence of 644 residues in twitchin) indicates that these proteins possess additional conserved functions.

Motif II and immunoglobulin-like domains

The superfamily of proteins with sequence homology to immunoglobulins has been divided into three sets, the V set, the C1 set and the C2 set²³. Motif II has homology to domains in the proteins of this superfamily, and greatest similarity to those in the proteins of the C2 set, a group that includes neural cell adhesion molecule (N-CAM), myelin associated glycoprotein (MAG), the receptor for platelet derived growth factor (PDGFR) and the Fc receptor (FcR) (Fig. 3c). The motif II consensus sequence matches 13 out of 25 residues in an immunoglobulin C2-set consensus sequence. The copies of twitchin motif II are more similar to one another than they are to other members of the immunoglobulin superfamily, presumably reflecting special functional constraints.

All previously recognized members of the immunoglobulin superfamily are cell surface or extracellular molecules, and yet twitchin (and csmMLCK) is totally intracellular. As might be expected for an intracellular molecule, the twitchin motif generally lacks the cysteines that form intra-chain disulphide bonds in immunoglobulin domains (copies 1' and 20' are the exception, in which both cysteines are found). The absence of these cysteines is not without precedent, because other members of the immunoglobulin superfamily have been found that lack the cysteines²³. For these exceptions and in most copies of twitchin motif II, hydrophobic residues reside at the positions of the conserved cysteines.

Twitchin motifs and cell adhesion molecules

Motif I of twitchin is similar to two tandem segments in the neural cell adhesion molecule (N-CAM)²⁴ (residues 479–578 and residues 580–673), with many of the twitchin consensus residues present at a spacing similar to that in the N-CAM copies (Fig. 2b). These portions of NCAM have similarity to segments in other cell adhesion molecules of insects and mice^{25,26}. This family of adhesion proteins has segments that are similar to the type III domains of fibronectin^{25,27}, as does twitchin motif I (especially over the last 33 residues of motif I). Twitchin, csmMLCK and these cell adhesion molecules contain both motifs I and II, but all other previously recognized members of the immunoglobulin superfamily have only motif II. It is striking that twitchin and the cell adhesion molecules N-CAM and fasciclin II each begin with five tandem copies of

MOTIF II

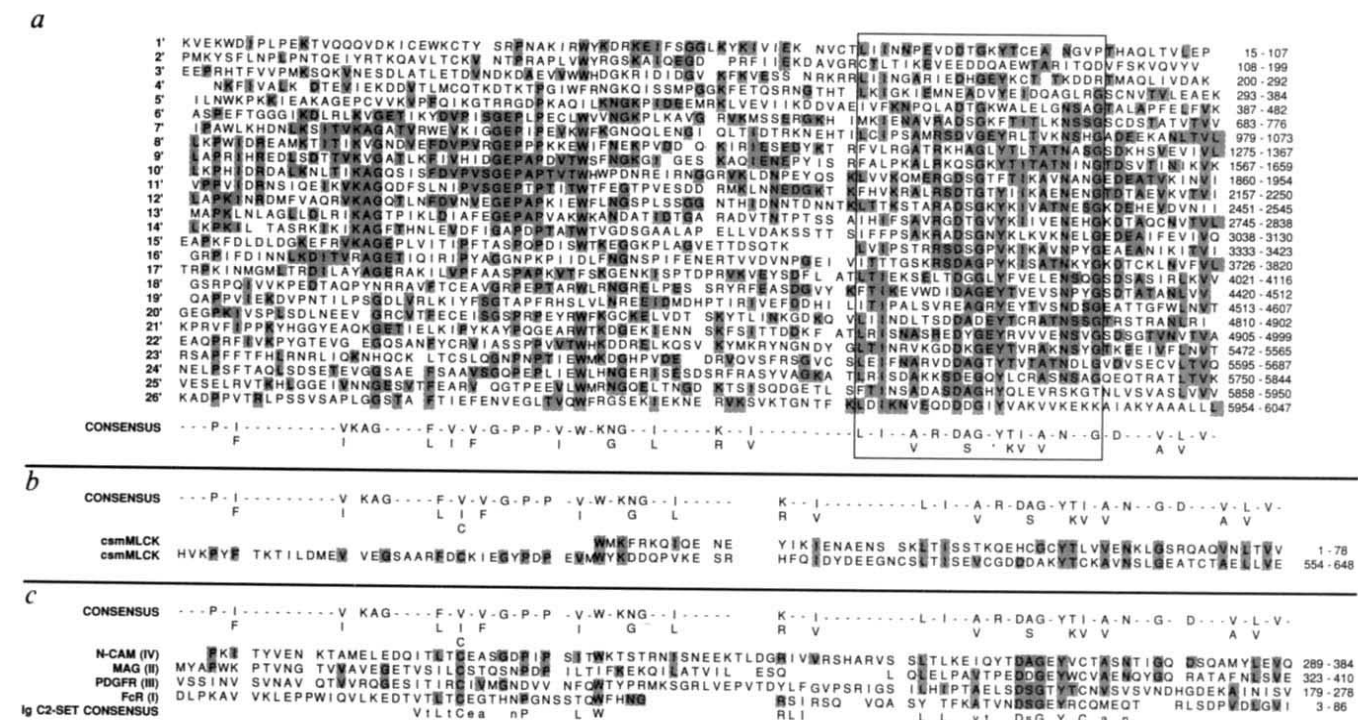


FIG. 3 Motif II (single-letter amino acid code). **a**, The 26 copies of motif II in twitchin. Numbers 1'–26' on the left correspond to the lightly shaded boxes in Fig. 1d. At each position, amino acids found in at least five copies are shaded. The consensus sequence was derived as residues present at corresponding positions in at least 9 out of 26 copies, or two residues that occur at corresponding positions between them at least 14 out of 26 copies. **b**, Similarity of motif II of twitchin to sequences in csmMLCK. Amino-acid residues identical to the consensus are shaded. The partial copy of motif II in csmMLCK matches the twitchin consensus at 15 positions and the complete copy of motif II in csmMLCK matches the twitchin consensus at

25 positions. **c**, The twitchin motif II consensus is compared with the following sequences of members of the C2 set of the immunoglobulin superfamily of proteins: N-CAM, fourth domain²⁴; myelin-associated glycoprotein (MAG), second domain⁴⁴; PDGFR, third domain⁴⁵, and the immunoglobulin Fc receptor (FcR) first domain⁴⁶. Residues identical to the twitchin consensus are shaded. Also displayed is a consensus for the immunoglobulin C2-set domains (modified from ref. 23). Upper-case letters denote the most strongly conserved amino acids, and the lower-case letters denote the less strongly conserved residues at various positions. A box encloses one region particularly highly conserved between motif-II copies.

immunoglobulin-like domains followed by two tandem copies of motif I.

Twitchin repeat structure in titin

By using our data, copies of both motif I and II have been found in titin, another protein of striated muscle (J. Trinick, personal communication). The sequence of a small titin cDNA clone indicates further that in titin the 2:1:2:1 organization of motifs I and II is also conserved. Titin, a polypeptide of M_r $2.5\text{--}3.0 \times 10^6$ (refs 8 and 9), is the third most abundant protein of the myofilament lattice of vertebrate striated muscle¹⁰, and is probably bound to thick filaments for much of its length¹¹. The presence of both motif I and II in a third muscle protein indicates that these motifs may be found more generally in thick-filament-associated proteins.

Electron microscopy of purified titin reveals long string-like structures with a beaded appearance^{12,13}. The centre-to-centre spacing of these 'beads' is estimated to be ~ 40 Å and their diameter estimated also to be ~ 40 Å (ref. 13). The motif II sequence could form at least some of these beads because, as

X-ray crystallography shows, the immunoglobulin domain is a cylinder ~ 40 -Å long²⁸.

Implications for twitchin function

A role for twitchin in the regulation of the contraction-relaxation cycle was indicated earlier by the mutant phenotype of constant subcellular twitching, which is exacerbated by choline agonists. The discovery of the homology of twitchin with myosin light-chain kinase indicates what this role might be. Myosin light-chain kinases phosphorylate the regulatory myosin light chain in a variety of animals²⁹, and there is evidence that in vertebrate smooth muscle and nonmuscle tissue this phosphorylation is required for initiating muscle contraction³⁰. As *C. elegans*³¹ (like *Ascaris lumbricoides*³²) probably has both thick and thin-filament regulation of contraction, the elimination of light-chain phosphorylation could result in an incompletely activated muscle, unable to develop or sustain normal contraction. That twitchin is substantially larger than csmMLCK, and that the motif organization of twitchin is similar to that of titin, suggests that twitchin has additional functions.

... I PPKYHGGYEAQKGET IELK I PYKAY PQGEAR	WMKFRKQ I QENEY I K I ENAENSSKL	45 csmMLCK
	WT K DGEK I ENNSKFS I TTDDKFATL	4966 twitchin
T I S S T K Q E H C G C Y T L V V E N K L G S R Q A Q V N L T V V D K P D P P A G T P C A S D I R S S S L T L S W Y G S		105 csmMLCK
R I S N A S R E D Y G E Y R V V E N S V G S D S G T V N V T V A D V P E P P R - F P I I E N I L D E A V I L S W K P P		5025 twitchin
S Y D G G S A V Q S Y T V E I W N S V D N K W T D L T T C R S T S F N V Q D L Q A D R E Y K F R V R A A N V Y G I S E P		165 csmMLCK
A L D G G S L V T N Y T I E K R E A M G G S W S P C A K S R Y T Y T I E Q L R A G K Q Y E F R I I A E N K H G Q S K P		5085 twitchin
S Q E S E V V K V G E K Q E E - - - - E L K E E E A E L S D D E G K E T E V N - - - - - Y Q T V T I N T E Q		210 csmMLCK
G E P T A P V L I P G D E R K R R R G Y D V D E Q G K I V R G K G T V S S N Y D N Y V F D I W K Q Y P Q P V E I K H D		5145 twitchin
K V S D V Y N I E E R L G S G K F G Q V F R L V E K K T G K V W A G K F F K A Y S A K E K E N I R D E I S I M N C L H H		270 csmMLCK
H V L D H Y D I H E E L G T G A F G V V H R V T E R A T G N N F A A K F V M T P H E S D K E T V R K E I Q T M S V L R H		5205 twitchin
P K L V Q C V D A F E E K A N I V M V L E M V S G G E L F E R I I D E D F E L T E R E C I K Y M R Q I S E G V E Y I H K		330 csmMLCK
P T L V N L H D A F E D D N E M V M I Y E F M S G G E L F E K V A D E H N K M S E D A V E Y M R Q V C K G L C H M H E		5265 twitchin
Q G I V H L D L K P E N I M C V N K T G T S I K L I D F G L A R R L E S A G S L K V L F G T P E F V A P E V I N Y E P I		390 csmMLCK
N N Y V H L D L K P E N I M F T T K R S N E L K L I D F G L T A H L D P K Q S V K V T T G T A E F A A P E V A E G K P V		5325 twitchin
G Y E T D M W S I G V I C Y I L V S G L S P F M G D N D N E T L A N V T S A T W D F D D E A F D E I S D D A K D F I S N		450 csmMLCK
G Y Y T D M W S V G V L S Y I L L S G L S P F G G E N D D E T L R N V K S C D W N M D D S A F S G I S E D G K D F I R K		5385 twitchin
L L K K D M K S R L N C T Q C L Q H P W L Q K D T K N M E A K K L S K D R M K K Y M A - - R R K W Q K T G H A V R A I G		508 csmMLCK
L L L A D P N T R M T I H Q A L E H P W L T P G N A P G R D S Q I P S S R Y T K I R D S I K T K Y D A W P E L P P L G		5445 twitchin
R L S S M A M I S G M S G R K A S G S S P T S P I N A D K V E N E D A F L E - E V A E E K P H V K P Y F T K T I L D M E		567 csmMLCK
R I S N Y S - - - - S L R K H R P Q E Y S I R - - - - - D A F W D R S E A Q P R F I V K P Y G T - - - - - E		5485 twitchin
V V E G S A A R F D C K I E G Y P D P E V M W Y K D D Q P V K E S R H F Q I D Y D E E G N C S L T I S E V C G D D D		625 csmMLCK
V G E G Q S A N F Y C R V I A S S P P V V T W H K D D R E L K Q S V K Y M K R Y N G N D Y G - L T I N R V K G D D K		5542 twitchin
A K Y T C A V N S L G E A T C T A E L L V E T M G K E G E G E G E G E E D E E E E E		669 csmMLCK
G E Y T V R A K N S Y G T K E E I V F L N V T R H S I P L K F E P L E P M K K A P S P P . . .		5586 twitchin

FIG. 4 Twitchin and csmMLCK homology (single-letter amino-acid code). Alignment was obtained by a modification of the Needleman and Wunsch⁴⁷ algorithm (ALIGN; Riverside Scientific Enterprises) and by inspection. Numbers correspond to positions in the deduced amino-acid sequences. The available sequence (about 60%) of csmMLCK²¹ is shown. Two dots indicate the same amino acid and one dot represents conservative substitutions. The box with the solid border denotes the 250-amino-acid regions of both sequences that are homologous to catalytic domains of protein kinases.

The box with the dotted border denotes motif I and the boxes with dashed borders boxes denote motif II. In the csmMLCK sequence, there is a single copy of motif I and one partial and one full copy of motif II. Twitchin has two short sequences, DLKPEN (residues 5,271-5,276) and GTAFAAPE (residues 5,309-5,317) that are very similar to sequences characteristic of serine/threonine kinases²⁰. There is no significant homology between the csmMLCK calmodulin-binding domain (ref. 21; underlined residues 480-510) and the corresponding region of twitchin.

Most probably twitchin associates with the thick filament, as it is found in the myosin-containing A bands and co-localizes with myosin in mutants in which muscle structure is abnormal⁷. The regular spacing and pattern of the motifs throughout much of the protein are well suited for the interaction with the repeated structure of the thick filament, which consists of a regular staggered array of myosin rods and paramyosin. Furthermore, both the myosin rod and paramyosin are composed of tandem copies of a 28-amino-acid motif estimated to be 42-Å long^{33,34}, a size similar to that of an immunoglobulin domain and perhaps motif II as well. But the regularity of the motifs is disrupted in several places, including two occurrences of motif-I triplets, one instance of a motif-I singlet, and two occurrences of motif II doublets (Fig. 1d). Whether these represent adaptations to fixed irregularities in thick filament structure or relaxed functional requirements of the protein is unclear; note that deletions in the gene that maintain the reading frame, yet disrupt the domain structure, can result in proteins with nearly normal function³⁵.

The association of the protein with the thick filament may localize the kinase domain near the myosin heads on the surface of the filament. This also means, however, that neither the kinase nor its substrate myosin can diffuse within the cell. Yet each kinase domain must act on many myosins, as there is estimated to be only one mole of twitchin per 10–50 moles of myosin.

Perhaps the kinase domain has enough flexibility to allow it to act on many myosins. Alternatively, the repeated domains themselves could play a part in regulation. If, indeed, the protein interacts along the long axis of the filament, it could serve to coordinate the activity of many myosins, in a manner analogous to the way in which tropomyosin regulates multiple actins. Also, like tropomyosin, the molecule could propagate changes in activity generated by the kinase domain down the filament, or calcium levels could directly alter the conformation of the repetitive part of the molecule. Consistent with these ideas is the finding that twitchin is required in near stoichiometric amounts; the loss of just half of the *unc-22* activity results in detectable twitching.

Does twitchin also have a fundamental role in thick filament assembly? The myofilament lattice is disrupted in nearly all *unc-22* mutants, but the numbers of thick filaments, even in the complete absence of twitchin, are about normal². Furthermore, the assembly of these filaments into a sarcomere can be normal in the absence of twitchin, provided that certain missense mutations are present in the myosin head region^{4,5}. Twitchin seems, therefore, to be unnecessary for either normal thick-filament formation or normal sarcomere assembly; a structural role for twitchin may yet, however, be found. We suggest instead that this unusually large protein has an important role in regulating the contraction-relaxation cycle. □

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- Waterston, R. H. in *The Nematode Caenorhabditis elegans* (ed. Wood, W.B.) 281–335 (Cold Spring Harbor Laboratory, New York, 1988).
- Waterston, R. H., Thomson, J. N. & Brenner, S. *Dev. Biol.* **77**, 271–302 (1980).
- Moerman, D. G. thesis, Simon Fraser Univ. Burnaby, British Columbia (1980).
- Moerman, D. G., Plurad, S., Waterston, R. H. & Baillie, D. L. *Cell* **29**, 773–781 (1982).
- Dibb, N. J. *et al.* *J. molec. Biol.* **183**, 543–551 (1985).
- Moerman, D. G., Benian, G. M. & Waterston, R. H. *Proc. natn. Acad. Sci. U.S.A.* **83**, 2579–2583 (1986).
- Moerman, D. G., Benian, G. M., Barstead, R. J., Schriever, L. A. & Waterston, R. H. *Genes Dev.* **2**, 93–105 (1988).
- Maruyama, K., Kimura, S., Yoshidomi, H., Sawada, H. & Kikuchi, M. *J. Biochem.* **95**, 1423–1433 (1984).
- Kurzban, G. P. & Wang, K. *Biochem. biophys. Res. Commun.* **150**, 1155–1161 (1988).
- Wang, K., McClure, J. & Tu, A. *Proc. natn. Acad. Sci. U.S.A.* **76**, 3698–3702 (1979).
- Whiting, A., Wardale, J. & Trinick, J. *J. molec. Biol.* **205**, 263–268 (1989).
- Wang, K., Ramirez-Mitchell, R. & Palter, D. *Proc. natn. Acad. Sci. U.S.A.* **81**, 3685–3689 (1984).
- Trinick, J., Knight, P. & Whiting, A. *J. molec. Biol.* **180**, 331–356 (1984).
- Wang, K. & Williamson, C. L. *Proc. natn. Acad. Sci. U.S.A.* **77**, 3254–3258 (1980).
- Koenig, M., Monaco, A. P. & Kunkel, L. M. *Cell* **53**, 219–228 (1988).
- Coulson, A., Sulston, J., Brenner, S. & Karn, J. *Proc. natn. Acad. Sci. U.S.A.* **83**, 7821–7825 (1986).
- Mori, I., Benian, G. M., Moerman, D. G. & Waterston, R. H. *Proc. natn. Acad. Sci. U.S.A.* **85**, 861–864 (1988).
- Emmons, S. W. in *The Nematode Caenorhabditis elegans* (ed. Wood, W. B.) 57–59 (Cold Spring Harbor Laboratory, New York, 1988).
- Blumenthal, T. & Thomas, J. *Trends Genet.* **4**, 305–308 (1988).
- Hanks, S. K., Quinn, A. M. & Hunter, T. *Science* **241**, 42–52 (1988).
- Guerriero, V., Russo, M. A., Olson, N. J., Putkey, J. A. & Means, A. R. *Biochemistry* **25**, 8372–8381 (1986).
- Takio, K., Blumenthal, D. K., Walsh, K. A., Titani, K. & Krebs, E. G. *Biochemistry* **25**, 8049–8057 (1986).
- Williams, A. F. & Barclay, A. N. *Rev. Immun.* **6**, 381–405 (1988).
- Cunningham, B. A. *et al.* *Science* **236**, 799–806 (1987).

- Harrelson, A. L. & Goodman, C. S. *Science* **242**, 700–708 (1988).
- Moos, M. *et al.* *Nature* **334**, 701–703 (1988).
- Kornblihtt, A. R., Umezawa, K., Vibe-Pedersen, K. & Baralle, F. E. *EMBO J.* **4**, 1755–1759 (1985).
- Schiffer, M., Girling, R. L., Ely, K. R. & Edmundson, A. B. *Biochemistry* **12**, 4620–4631 (1973).
- Edelman, A. M., Blumenthal, D. K. & Krebs, E. G. *A. Rev. Biochem.* **56**, 567–613 (1987).
- Kamm, K. E. & Stull, J. T. *A. Rev. Pharmacol. Tox.* **25**, 593–620 (1985).
- Harris, H. E., Tso, M.-Y. W. & Epstein, H. F. *Biochemistry* **16**, 859–865 (1977).
- Lehman, W. & Szent-Gyorgyi, A. G. *J. gen. Physiol.* **66**, 1–30 (1975).
- McLachlan, A. D. & Karn, J. *Nature* **299**, 226–231 (1982).
- Kagawa, H., Gengyo, K., McLachlan, A. D., Brenner, S. & Karn, J. *J. molec. Biol.* **207**, 311–333 (1989).
- Kiff, J. E., Moerman, D. G., Schriever, L. A. & Waterston, R. H. *Nature* **331**, 631–633 (1988).
- Moerman, D. G. & Baillie, D. L. *Genetics* **91**, 95–103 (1979).
- Moerman, D. G. & Waterston, R. H. *Genetics* **108**, 859–877 (1984).
- Rogalski, T. M. & Baillie, D. L. *Molec. Gen. Genet.* **201**, 409–414 (1985).
- Bankier, A. T. & Barrell, B. G. in *Techniques in the Life Sciences* Vol. B508 (ed. Flavell, R. A.) 1–34 (Elsevier, Ireland, 1983).
- Sanger, F., Nicklen, S. & Coulson, A. R. *Proc. natn. Acad. Sci. U.S.A.* **74**, 5463–5467 (1977).
- Tabor, S. & Richardson, C. C. *Proc. natn. Acad. Sci. U.S.A.* **84**, 4767–4771 (1987).
- Ansorge, W. & Labeit, S. *J. Biochem. biophys. Meth.* **10**, 237–243 (1984).
- Staden, R. *Nucleic Acids Res.* **14**, 217–231 (1986).
- Arquint, M. *et al.* *Proc. Acad. Sci. U.S.A.* **84**, 600–604 (1987).
- Yarden, Y. *et al.* *Nature* **323**, 226–232 (1986).
- Lewis, V. A., Koch, T., Plutner, H. & Mellman, I. *Nature* **324**, 372–375 (1986).
- Needleman, S. B. & Wunsch, D. C. *J. molec. Biol.* **48**, 545–552 (1970).

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Is Cygnus X-3 blowing bubbles?

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CYGNUS X-3, one of the brightest X-ray sources in the Galaxy and perhaps also a source of very-high-energy (TeV) photons or particles, is thought to be a close binary system (orbital separation $\sim 10^{11}$ cm and period 4.8 h) whose emission is powered by accretion of mass from a main-sequence star like the Sun onto a neutron star, or possibly a black hole. The X-ray luminosity, $L_x \sim 10^{38}$ erg s $^{-1}$, implies a radiation rate near the Eddington limit. Recent radio observations of Cyg X-3 (ref. 1) have revealed extended radio lobes on a scale of ~ 0.3 pc, elongated in the same direction in which clouds of relativistic plasma, discovered by very-long-baseline interferometry observations $^{2-4}$ during radio outbursts, are ejected from the binary system. I argue here that the extended radio lobes can be understood as bubbles blown into a dense interstellar medium by the cumulative energy of the bursts from the central object. If this is correct, the extended lobes of Cyg X-3 would have to be $\sim 2,000$ years old.

At radio frequencies, Cyg X-3 shows strong, irregularly spaced bursts of emission in which the flux rises by a factor of 100–1,000 above the typical level 5 . The mean interval between bursts is $p_b \sim 120$ days. The bursts can be interpreted as synchrotron radiation from an expanding cloud of relativistic electrons in a magnetic field of 10^{-2} G. Very-long-baseline interferometry (VLBI) observations $^{2-4}$ during bursts show an expanding elongated radio-frequency source on a scale of 10^{14} cm. Assuming a distance to Cyg X-3 of 10 kpc, the projected expansion velocity is $V_{exp} \approx 0.3\text{--}0.4$ c. The minimum energy per burst, U_b , required to power the emission is about 10^{44} erg (ref. 7), and the associated average luminosity L_b is $U_b/p_b \approx 10^{37}$ erg s $^{-1}$, which is about $0.1 L_x$. The recently discovered 1 extended radio lobes have a linear extent of $D \approx 10^{18}$ cm. The extended lobes are elongated in the direction of the VLBI-detected expansion, which suggests a causal connection with the bursts.

The physical properties of the extended lobes calculated assuming that the radio emission is synchrotron radiation are summarized in Table 1. The minimum energy, U_{min} , stored by relativistic electrons and magnetic fields in the lobes is estimated to be $\sim 10^{45}$ erg. This energy could be supplied by the bursts in a time $t_{min} \approx U_{min}/L_b \approx 3$ yr. This energy could be supplied by the bursts in a time $t_{min} \approx U_{min}/L_b \approx 3$ yr. This is of the same order of magnitude as the dynamical timescale $t_{dyn} \approx D/V_{exp}$ derived from the VLBI-detected source expansion. In contrast, the radiation-loss time of the synchrotron electrons, t_{loss} , is $\sim 10^6$ yr. The fact that $t_{loss} \gg t_{dyn}$, t_{min} suggests that most of the energy supplied to the lobes by the central source is expended in driving the expansion of the lobes, rather than in building up the internal energy density and compensating for synchrotron losses. In that case, the lobes must be much older than the simple estimate t_{min} would suggest.

I propose a model for the extended lobes that is analogous to the formation of bubbles in the interstellar medium by stellar winds. It differs from conventional models of radio-lobe formation used in the context of active galaxies and quasars in the assumption that the momentum flux of the outflow is not well collimated as in a jet with constant cross-section, but instead is distributed over an area scaling as D^2 , where D is the linear extent of the source. This would be the case for instance in a conical jet with a fairly large constant opening angle, or in a quasi-spherical expansion.

On timescales $t \gg p_b$ the expansion is driven by the cumulative energy of the bursts. A self-similar solution describes a shock propagating into the interstellar medium with typical radius 8 $R(t) = \alpha(L_b/\rho)^{1/5} t^{3/5}$, where ρ is the density of the interstellar medium, t is the age of the source and α is a numerical constant

of the order of unity. The hot interior of the bubble and the shocked interstellar medium are separated by a contact discontinuity at radius $R_{cd} \approx 0.83 R(t)$. For spherical symmetry (opening angle near 4π), $\alpha \approx 0.88$ during the initial adiabatic phase of the expansion and $\alpha \approx 0.76$ after the shell of swept-up interstellar medium has collapsed through internal cooling and radiates efficiently. The detailed shape of the lobe depends on the (unknown) angular distribution of the momentum flux driving the expansion, and can be calculated using a generalized Kompaneets approximation 9,10 . The lobes will be elongated along the direction of the beamed outflow from the central source, as observed.

Given the observed size, D , of the extended lobes, one can use the above expansion law to estimate the age of the source:

$$t \text{ (yr)} \approx 1.5 \times 10^3 \alpha^{-5/3} D_{18}^{5/3} n_2^{1/3} L_{37}^{-1/3} \quad (1)$$

where D_{18} is the linear size of the source in units of 10^{18} cm. n_2 is the number density in the interstellar medium in units of 100 cm^{-3} and L_{37} is the driving luminosity in units of 10^{37} erg s $^{-1}$. The age of a few thousand years for the extended source is much less than the evolutionary timescale for a close binary system ($\geq 10^6$ yr). This suggests that the bursts driving the expansion are a fairly recent phenomenon in Cygnus X-3. The expansion velocity of the lobes is $\dot{D} = 1.3 \times 10^2 \alpha^{8/3} L_{37}^{1/3} n_2^{-1/3} D_{18}^{-2/3} \text{ km s}^{-1}$, and the internal pressure in the lobes is roughly equal to the pressure behind the strong shock, $P_s \approx (3/4)\rho\dot{D}^2 \approx 2 \times 10^{-8} \alpha^{16/3} n_2^{1/3} L_{37}^{2/3} D_{18}^{-4/3} \text{ erg cm}^{-3}$. This yields a total thermal energy U_{th} of the lobes of the order of $(3/2)P_s \times (4\pi D^3/3) \approx 10^{47} \alpha^{16/3} L_{37}^{2/3} n_2^{1/3} D_{18}^{5/3} \text{ erg}$. Comparing U_{th} with the minimum energy U_{min} needed to explain the extended synchrotron emission, I estimate that $\sim 1\%$ of the internal energy must reside in the relativistic electrons and magnetic fields responsible for the synchrotron emission. The total energy U_{tot} supplied to the lobes, including the kinetic energy of the swept-up interstellar material, is $L_b t = 4.5 \times 10^{47} \alpha^{-5/3} D_{18}^{5/3} L_{37}^{2/3} n_2^{1/3} \text{ erg} \approx 4.5 \times 10^2 \alpha^{-5/3} U_{min}$.

In its lifetime the extended source has swept up interstellar gas with total mass $M_s(g) \approx (4\pi/3)\rho D^3 \approx 10^{33} n_2 D_{18}^3$. The swept-up gas directly behind the shock has a temperature $T_+ \text{ (K)} \approx (3\mu m_H/16k_B)\dot{D}^2 = 5 \times 10^5 \alpha^{16/3} L_{37}^{2/3} n_2^{-2/3} D_{18}^{-4/3}$, where $\mu \approx 1.4$ is the mean relative molecular mass per hydrogen atom, m_H is the mass of a hydrogen atom and k_B is the Boltzmann constant. Radiation losses per unit volume $C(T)$ in this hot gas of ionized hydrogen and multiply ionized other elements are $n_e n_p \Lambda(T)$, where the cooling function $\Lambda(T) \approx 1.6 \times 10^{-22} T_6^{-0.6} \text{ erg cm}^3 \text{ s}^{-1}$, T_6 being the temperature of the gas in units of 10^6 K. Here n_e and n_p are the electron and proton densities. Cooling is primarily due to line emission for $T \leq 10^7$ K. Defining a net compression ratio $n_s/n \equiv r$ for the swept-up gas and taking $n_e \approx n_p \approx n_s$, the typical cooling time behind the shock, $t_c \approx 3n_s k_B T / (2C(T)) \approx 1.5 \times 10^2 (T_6)^{1.7} r^{-1} n_2^{-1} \text{ yr}$. This is shorter than the age of the lobes provided that $rn_2 \geq 0.1$, so that it is likely that the shocked interstellar material has collapsed into a thin shell, or is in the process of doing so.

The energy advected into the shell across the shock per unit time, $L_s \approx 4\pi D^2 \times (\rho\dot{D}^2/2) \approx 2\pi\alpha^2(3/5)^3 L_b \approx \epsilon L_b$, is largely radiated away ($\epsilon \approx 0.3$ if $\alpha \approx 0.76$, and L_s is the luminosity of the shocked material). The hot layer of ionized hydrogen and multiply ionized ions cools by radiating most of its energy in the ultraviolet at wavelengths $< 912 \text{ \AA}$. At a column density of $N \approx 10^{17} \text{ cm}^{-2}$ behind the shock, the temperature reaches $T \approx 10^4$ K and a recombination zone begins which reabsorbs this ionizing ultraviolet radiation. This zone therefore radiates with a total luminosity of about L_s , cooling through optical and ultraviolet radiation 11,12 . A typical cooling rate has $\Lambda_{rec} \approx 2 \times 10^{-23} \text{ erg cm}^3 \text{ s}^{-1}$ (refs 13, 14). The thermal free-free emission from the dense shell comes primarily from the recombination zone. The free-free emissivity per unit volume is $n_e n_p \Lambda_{ff}(T)$, with a free-free cooling function $\Lambda_{ff}(T) \approx 2.4 \times 10^{-25} T_4^{1/2} \text{ erg cm}^3 \text{ s}^{-1}$, where T_4 is the temperature of the

recombination zone in units of 10^4 K. The total free-free luminosity can be estimated as $L_{\text{ff}} \approx \Lambda_{\text{ff}}(T) \epsilon L_b / (\Lambda_{\text{rec}} + \Lambda_{\text{ff}}(T)) \approx 4 \times 10^{34} T_4^{1/2} (\epsilon/0.3) L_{37} \text{ erg s}^{-1}$. The free-free emission from the hot region directly behind the shock is about a factor of 100 less intense. At low frequencies ($h\nu \ll k_B T$) the monochromatic free-free emission flux (S_ν) at the Earth for an assumed distance of $d = 10$ kpc is about $(h/k_B T)(L_{\text{ff}}/4\pi d^2) \approx 2(\epsilon/0.3) L_{37} \text{ mJy}$. Extinction can be neglected at a wavelength of 6 cm. This is about a factor of two less than the observed extended flux density. It therefore seems likely that a significant part of the radio emission is synchrotron radiation, as was assumed by Strom *et al.*¹. This interpretation is supported by their detection of (partial) polarization of the 6-cm emission. The relative contribution of thermal free-free radiation will remain uncertain until multi-frequency observations determine the spectrum of the radio emission from the extended lobes.

The relativistic electrons filling the lobes have probably not been produced directly by the bursts. A power-law distribution of relativistic electrons— $(dN/d\gamma) \propto \gamma^{-s}$ where γ is the Lorentz factor (integrated over volume)—that are created at an individual burst in a volume V_0 , carrying a fraction f of the total burst energy U_b , contributes a relativistic-electron energy $U_e = f U_b (V/V_0)^{-(s-1)/3}$ after adiabatic expansion into the volume V of the extended lobes. The VLBI-detected clouds have a volume $V_0 \leq 10^{45} \text{ cm}^3$, whereas the observed spectrum is consistent with $s \approx 2$. After expansion into the whole lobe volume $V \approx 10^{54} \text{ cm}^3$, the remaining relativistic-electron energy per burst is $U_e \leq 10^{-3} f U_b \approx 10^{41} f (U_b)_{44} \text{ erg}$ (where $(U_b)_{44}$ is the burst energy in units of 10^{44} erg). This is four orders of magnitude less than the relativistic-electron energy present in the lobes if the observed 6-cm emission has a significant non-thermal component. This excludes the possibility that the extended lobes are adiabatically expanding, optically thin synchrotron clouds created by a single large burst in the central object about three years ago. The energy of the cloud accumulated from all the bursts in a time $t_{\text{loss}} > t \gg p_b$ is $U_c \approx (t/p_b) U_e = f(V/V_0)^{-(s-1)/3} L_b t = 10^{-3} f U_{\text{tot}}$. For f of the order of unity, $U_c \approx U_{\text{min}}$, but it seems unlikely that all the energy in the bursts resides in relativistic electrons.

The total monochromatic synchrotron luminosity of the lobes that is due to the accumulated relativistic electrons is $S_\nu = (t/p)(B/B_0)^{(s+1)/2} (V/V_0)^{-(s-1)/3} S_\nu(\text{burst})$. The observations of the bursts are consistent with $B_0 \approx 0.1$ G, whereas the lobes have $B_{\text{eq}} \approx 10^{-4}$ G. Here B_0 , B and B_{eq} are the magnetic field in the VLBI clouds, the extended lobes and the equipartition field respectively. This gives $S_\nu \approx 10^{-4} S_\nu(\text{burst}) \leq 1 \text{ mJy}$, as the strongest bursts have $S_\nu(\text{burst}) \approx 10 \text{ Jy}$. This is at least a factor of five less than the observed flux density (see Table 1). Some parameters, however, are quite uncertain—in particular the energy density in the lobes and the magnetic field strength B , which are derived from the minimum-energy assumption. These estimates imply that some reacceleration and/or fresh production of relativistic electrons is required in the lobes to partly compensate for expansion losses, which increases the synchrotron luminosity.

An efficient acceleration mechanism that is likely to operate in the lobes is diffusive shock acceleration¹⁵. Particles will be accelerated at the strong exterior shock that encloses the lobes or at shocks generated when clouds formed at each burst collide with the dense shell of shocked interstellar material behind that exterior shock. The mean energy gain in this process of a particle with Lorentz factor γ , diffusing with spatial diffusion coefficient K , at a strong shock with propagation velocity u_s is

$$\frac{1}{\gamma} \left(\frac{d\gamma}{dt} \right) \approx \frac{u_s^2}{4\langle K \rangle} \quad (2)$$

Here $\langle K \rangle$ is a mean spatial diffusion coefficient, defined by $\langle K \rangle = K_- + 4K_+$. The subscripts $-$ and $+$ denote typical values in the medium upstream and downstream of the shock. The relativistic electrons diffuse by gyro-resonant scattering from

TABLE 1 Observational parameters

Distance Cyg X-3: 10 ± 2 kpc.
X-ray luminosity: $L_x = 10^{38} \text{ erg s}^{-1}$
Mean 6-cm flux density associated with burst: $S_\nu(\text{burst}) \approx 5 \text{ Jy}$
Extended 6-cm flux density at Earth: $S(\nu = 4.9 \text{ GHz}) = 4.5 \text{ mJy}$
Radio luminosity of extended lobes: $L_r = 3 \times 10^{30} \text{ erg s}^{-1}$
Diameter of extended lobes: $D = 0.3 \text{ pc} = 10^{18} \text{ cm}$
Minimum energy: $U_{\text{min}} = 10^{45} \text{ erg}$
Equipartition field: $B_{\text{eq}} = 10^{-4} \text{ G}$

magneto-hydrodynamic waves. The associated diffusion coefficient is $K = (cr_g/3) I(k_{\text{res}})^{-2}$, where $r_g = \gamma m_e c^2 / eB = 1.6 \times 10^7 \gamma B_{-4}^{-1} \text{ cm}$ is the gyroradius of an electron with Lorentz factor γ and $I(k_{\text{res}}) \equiv \delta B(k_{\text{res}})/B$ is the relative magnetic amplitude of the waves at the resonant wavenumber $k_{\text{res}} \approx 1/r_g$. For strong turbulence ($\delta B(k)/B \approx 1$ at all scales), one has $K = K_0 \gamma$, where $K_0 = m_e c^3 / 3eB \approx 10^{18} B_{-4}^{-1} \text{ cm}^2 \text{ s}^{-1}$ is the diffusion coefficient of a particle with $\gamma \approx 1$. Electrons injected into the acceleration region with $\gamma \approx 1$ gain energy according to $\gamma(t) \approx 1 + u_s^2 t / (4\langle K_0 \rangle)$. This means that electrons radiating by the synchrotron mechanism at $\lambda = 6 \text{ cm}$ with a shock Lorentz factor $\gamma_s \approx 1,000$ can be accelerated in a time $t_{\text{acc}} \approx 8\gamma_s K_0 / u_s^2 \approx 10^6 (K_0)_{18} / (u_s)_8^2 \text{ s}$, where I have assumed that $(K_0) \approx 2K_0 ((u_s)_8)$ is the shock propagation velocity in units of 10^8 cm s^{-1} and $(K_0)_{18}$ is the spatial diffusion coefficient in units of $10^{18} \text{ cm}^2 \text{ s}^{-1}$. Acceleration at the outer shock with $(u_s)_8 \approx 0.1$ can proceed to the required energies in $\sim 3(K_0)_{18} \text{ yr}$. This is much less than the age of the source provided $K_0 \leq 10^{21} \text{ cm}^2 \text{ s}^{-1}$, allowing for less-efficient scattering by weak turbulence with $\delta B(k_{\text{res}})/B \approx 0.03$. Alternatively, if the central source ejects clouds moving ballistically, as does SS433¹⁶, these clouds will collide with the outer dense shell of the lobes. This will also result in the formation of shocks when these individual clouds break up. With an assumed cloud velocity $V_c \approx V_{\text{exp}} \approx 0.35c$ and a cloud size r_c , the maximum time available for acceleration at such a collision is $r_c/V_c \approx 10^6 (r_c)_{16} \text{ s}$ (where $(r_c)_{16}$ is r_c in units of 10^{16} cm). As u_s is expected to be of the order of $V_{\text{exp}} \sim 10^{10} \text{ cm s}^{-1}$, this is more than enough to (re)accelerate the electrons to the required GeV energies. It therefore seems likely that the modest amount of reacceleration required in this model can be supplied by diffusive shock acceleration, even with a low level of turbulence $\delta B(k_{\text{res}})/B \approx 0.1$. The maximum energy that can be attained by electrons from continuously operating shock acceleration at the outer shock is determined by synchrotron losses. Writing these losses as $(d\gamma/dt)_{\text{synch}} = -\gamma^2/\tau$, with $\tau = 6\pi m_e c / \sigma_T B^2 = 2 \times 10^9 B_{-4}^{-2} \text{ yr}$ (here σ_T the Thomson cross-section) and balancing them with the energy gain that is due to shock acceleration (equation (2)) (assuming as before that $\langle K \rangle = 2K_0 \gamma$) I obtain $\gamma_{\text{max}} \approx (u_s^2 \tau / 8K_0)^{1/2} \approx 10^7 (u_s)_8 B_{-4}^{-1} (K_0)_{18}^{1/2}$. Expansion losses in the self-similar flow $(d\gamma/dt)_{\text{exp}} \approx -(\dot{D}/D)\gamma \approx -3\gamma/5t$ become important at $\gamma \approx 5u_s^2 t / 24K_0 \approx 10^8 (u_s)_8^2 \alpha^{-5/3} D_{18}^{5/3} n_2^{1/3} L_{37}^{-1/3} (K_0)_{18}^{-1}$. □

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1. Strom, R. G., van Paradijs, J. & van der Klis, M. *Nature* **337**, 234–236 (1989).
2. Geldzahler, B. J. *et al. Astrophys. J.* **273**, L65–L69 (1983).
3. Spencer, R. E. & Johnston, K. J. in *RS Ophiuchi (1985) and the Recurrent Nova Phenomenon* (ed. Bode, F.) 215–219 (VNU Science Press, Utrecht, 1986).
4. Molnar, L. A., Reid, M. J. & Grindley, J. E. *Astrophys. J.* **331**, 494–508 (1988).
5. Bonnet-Bideaud, J. M. & Chardin, G. *Phys. Rep.* **170**, 325–404 (1988).
6. Johnston, K. J. *et al. Astrophys. J.* **309**, 707–713 (1986).
7. Seaquist, E. R. *Astrophys. J.* **207**, 88–95 (1976).
8. Weaver, R., McCray, R., Castor, J., Shapiro, P. & Moore, R. *Astrophys. J.* **218**, 377–395 (1977).
9. Icke, V. *Astr. Astrophys.* **202**, 177–188 (1988).
10. Bisnovaty-Kogan, G. S. & Blinnikov, S. I. *Soviet Astr.* **26**, 530–537 (1983).
11. Curiel, S., Cantó y Luis, J. & Rodríguez, L. F. *Rev. Mex. Astr. Astrofis.* **14**, 595–602 (1987).
12. Cox, D. P. & Raymond, J. C. *Astrophys. J.* **298**, 651–659 (1985).
13. Shull, J. M. & McKee, C. F. *Astrophys. J.* **227**, 131–149 (1979).
14. Hartigan, P., Raymond, J. & Hartmann, L. *Astrophys. J.* **316**, 323–348 (1987).
15. Blandford, R. D. & Eichler, D. *Phys. Rep.* **154**, 1–75 (1987).
16. Vermeulen, R. C., Schilizzi, P. T., Icke, V., Fejes, I. & Spencer, R. E. *Nature* **328**, 309–313 (1987).

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Ultra-low-frequency variability in a simple atmospheric circulation model

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To assess the inherent variability of atmospheric flow, we have carried out extended runs of a primitive-equation model of the global troposphere. The longest-period forcing that we explicitly included was the annual cycle, yet the largest-scale structures in the flow were variable on much longer timescales, with maximum variability on periods of 10–40 years. This variability was generated internally by the nonlinear dynamics of the atmospheric flow. Forcings by slow variations of such quantities as sea surface temperature or incoming solar radiation were excluded. The results show that apparent correlations with the sunspot cycle^{1–3} could be spurious. They also indicate that attempts to monitor systematic climate change require circulation statistics for periods longer than 40 years, and that studies of climate sensitivity based on global circulation models require integrations of at least that length.

The observed variability of atmospheric flow at low frequencies may be due either to the inherent, internally generated unsteadiness of that flow, or to external influences in the form of varying boundary forcing, changing solar radiation or to changes of atmospheric composition. The relative contribution of internal and external sources of variability is still uncertain. Arguments based on linear theories of instability and on the known limits of deterministic predictability suggest that internal variability dominates for periods less than 10–20 days. It is frequently assumed that external causes must be sought for lower-frequency variations. Certainly there has been a degree of success in relating part of the observed interannual variance to slowly varying patterns of sea surface temperature. The non-linearity of atmospheric flow does not necessarily mean, however, that its behaviour is completely random on the longer timescale. Coherent and relatively long-lived structures can arise. Extreme regularity in highly nonlinear flow is well known in experiments on rotating convecting fluids⁴, and long-lived coherent blocking patterns are observed in the atmosphere⁵. Here we show that internally generated variations of much lower frequencies than have previously been assumed dominate the spectrum of planetary-scale structures in an atmospheric model. The results imply that a degree of regularity underlies the apparently random fluctuations of the circulation.

We used a global version of the spectral primitive-equation model described in ref. 6. We used five equal mass layers in the vertical. The smallest horizontal scale included was equivalent to 21 wavelengths around the globe (wavenumber 21); this implies a smallest resolved scale of ~700 km, and is similar to the resolution of many operational global-circulation models. The model handles the nonlinear dynamics of the atmosphere in a relatively sophisticated manner, representing the large-scale circulation systems of both the tropics and mid-latitudes explicitly.

We modelled the effects of diabatic heating and friction by simple linear terms so that we could carry out extremely long integrations at comparatively modest cost. The atmospheric circulation was driven by a term in the thermodynamic equation that relaxed the temperature towards a 'radiative-convective equilibrium' on a timescale of 15 days. The solstice distribution of the equilibrium temperature is shown in Fig. 1a. Such a state is unstable, particularly to baroclinic instabilities in the middle latitudes which convert potential energy associated with the meridional temperature gradients into the kinetic energy of the atmospheric circulation. This energy conversion is eventually

balanced by the destruction of kinetic energy by friction at the Earth's surface. We modelled friction by a simple 'Rayleigh drag law' in the lowest model layer. A force $-\mathbf{v}/\tau_d$ acts on the air in the lowest layer, where $\mathbf{v}$ is the horizontal wind vector and τ_d a timescale, which we chose to be one day. We varied the pole-pole radiative equilibrium temperature difference sinusoidally to represent the seasonal cycle. The model included no moisture (except implicitly, by the definition of the radiative-convective equilibrium state) and the lower surface was uniform with no mountains or heat sources and sinks.

The mean temperature structure at the solstices is shown in Fig. 1b. The atmospheric circulation has increased the static stability and reduced the meridional temperature gradients by transporting heat upwards and polewards. Further discussion of the mean global circulation of this model and of its sensitivity to the imposed parameters is given in ref. 6.

The variability of the large-scale atmospheric flow is illustrated by spectra showing the frequency variation of certain coefficients of the spherical harmonic Y . We obtained the spectra by saving the spherical harmonic coefficients averaged with respect to pressure, every 10 (model) days, and applying a fast Fourier transform to the data for the last 96 years of the run. We excluded the first few years of the run to eliminate any initial transient effects. Figure 2a shows the spectrum of the Y_1^0 temperature coefficient. This is a measure of the inter-hemispheric

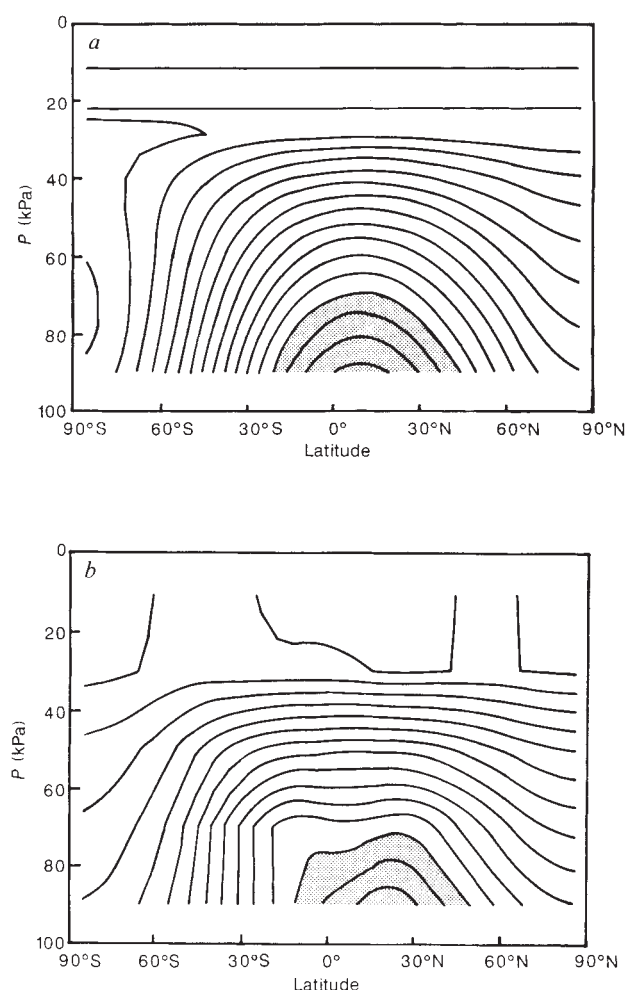


FIG. 1 Latitude-pressure cross-sections showing the zonal mean temperature distribution in *a*, the radiative-convective equilibrium state at the solstices and *b*, the model-generated solstice climatology. Contour interval 5 K, values in excess of an arbitrary reference temperature shaded.

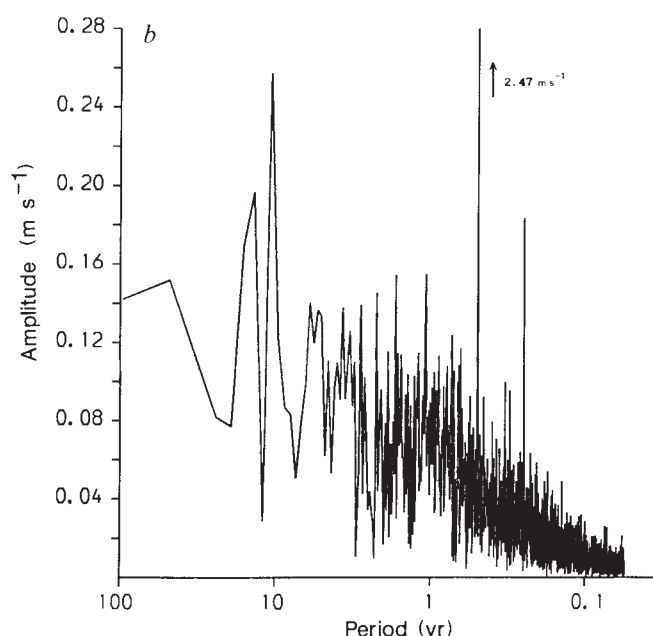
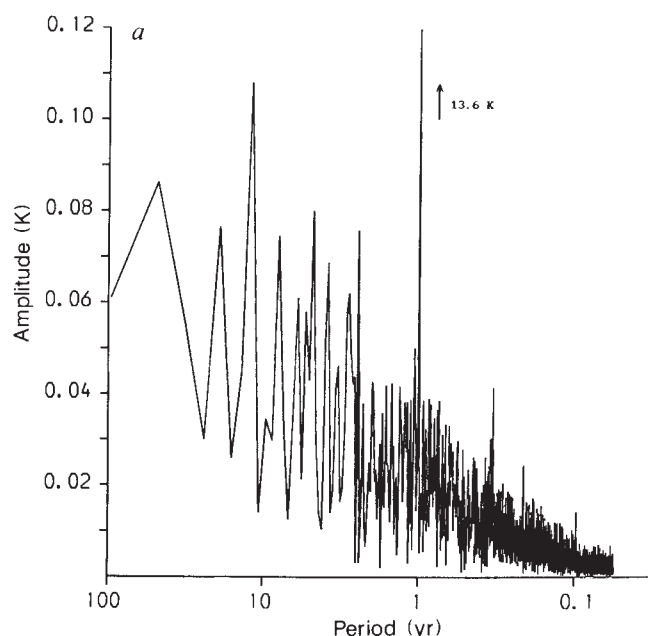


FIG. 2 Spectrum of the time variations of two selected spherical harmonic coefficients during the last 96 years of the model integrations. *a*, The Y_1^0 temperature coefficients, averaged with respect to pressure, giving the

mean pole-pole temperature difference. *b*, The Y_1^0 vorticity coefficient, averaged with respect to pressure, giving the mean solid-body rotation component of the atmospheric motion relative to the Earth.

temperature contrast. The forcing was applied to this spherical harmonic component to simulate the annual cycle, which introduced a sharp peak at a period of one year. We might have expected baroclinic instability to lead directly to a large variance on the 1–7 day timescale but, after the annual cycle, ultra-low frequencies had the largest amplitudes in the spectrum. The maximum was for a period of 12 years. Figure 2*b*, shows the spectrum of the Y_1^0 vorticity coefficient which measures the solid-body rotation component of the atmospheric motion relative to the Earth. This coefficient was not forced directly, but varied as the temperature variations set the model atmosphere into motion. The seasonal cycle was expressed by a strong peak at a period of six months, reflecting a maximum of atmospheric angular momentum at each equinox. Apart from this sharp seasonal peak, the strongest variability was at ultra-low frequency; the largest peak corresponded to a period of ten years.

The dominance of these ultra-low frequencies is marked for the largest-scale structures in the zonal mean flow. Smaller-scale structures have maximum variability on timescales which are progressively shorter, although still long compared with the 1–7 day timescale. Such timescales are frequently referred to as 'low frequencies' (for example, see ref. 7). Figure 3 shows the spectrum of surface temperature variations at a single point at 47° N. The signal is dominated by the passage of developing and decaying mid-latitude cyclones over the grid point. There is also a sharp peak with a period of 93 days. This is associated with the development of an equatorially trapped Kelvin wave of zonal wavenumber 1. This is an unrealistic feature of the present version of the model, and is due to the small dissipation of wavenumber 1 in the upper troposphere. Evidence from this run and runs with rather greater dissipation levels suggest that this mode does not interact significantly with the zonal mean flow, the fluctuations of which are shown in Fig. 2.

We have verified that the peaks in the Y_1^0 temperature and vorticity coefficient spectra are not simply a spurious result of the finite data record. We repeated the analysis with the mean annual cycle removed, and re-applied it with a Hanning window⁸

to eliminate end effects. The peaks at 10 and 12 years remain at more or less the same amplitude in all cases.

The seasonal cycle, which is the longest externally imposed timescale on the system, does not modify the ultra-low frequency behaviour significantly. A second 100-yr run was carried out, in which the radiative equilibrium temperature was fixed at its solstice values. The largest variability was at somewhat longer periods of 16 years with an amplitude comparable to that for the annual-cycle run.

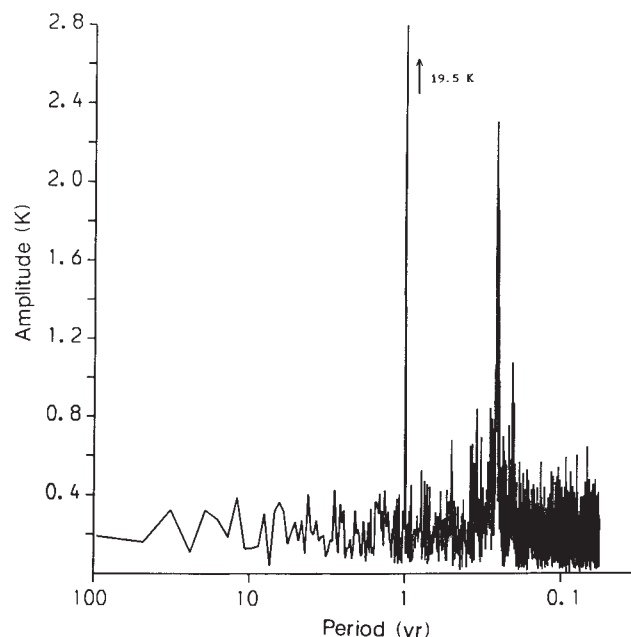


FIG. 3 Spectrum of the time variations of the surface temperature at a grid point at 47°N in the model.

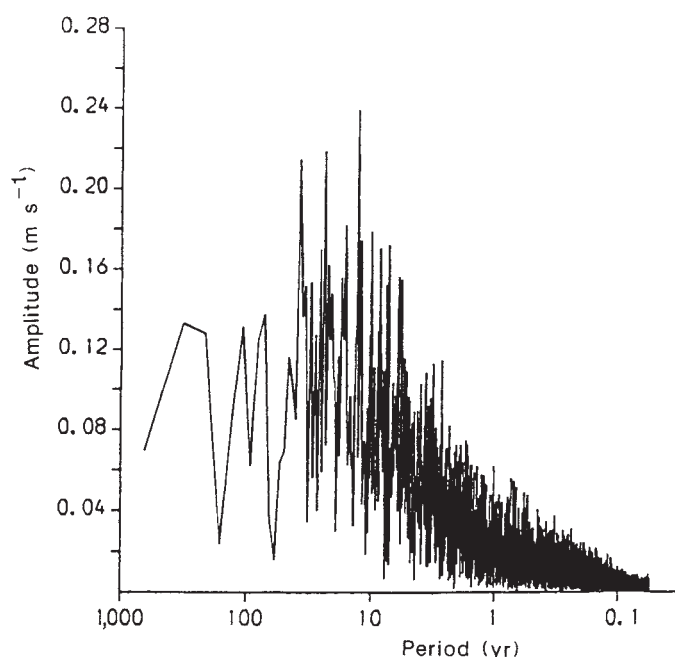


FIG. 4 As Fig. 2b, but for the 648-yr constant solstice calculation.

We fitted an autoregressive model to the time series of Y_1^0 coefficients. A 'red-noise' spectrum, with maximum variability at zero frequency gave the best fit. Standard statistical tests demonstrate the sharp peaks close to the period of the solar cycle are not *a posteriori* statistically different from the red-noise background. Rather, the spectra seem to be characterized by broad peaks with decadal periods. We extended the constant-solstice run to 648 years to test this suggestion. The spectrum of the Y_1^0 vorticity coefficient is shown in Fig. 4. A number of rather sharp peaks are seen in the range 10–40 years; the smoothed spectrum peaks in this range, and falls off towards both lower and higher frequencies. The maximum variability is clearly located within this range of periods.

The internal variability of the zonal mean part of the atmospheric circulation is large, on timescales as long as are currently available in accurate global data sets or in runs of global-circulation models. There is no reason to appeal to forcings external to the atmosphere (for example, solar forcing) to account for ultra-low-frequency variability. Models such as ours, with sophisticated atmospheric dynamics but simplified representation of other processes, are a benchmark against which to measure the ultra-low-frequency variability of more complex climate models. □

Critical currents near 10^6 A cm^{-2} at 77 K in neutron-irradiated single-crystal $\text{YBa}_2\text{Cu}_3\text{O}_7$

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THE defects in as-grown bulk $\text{YBa}_2\text{Cu}_3\text{O}_7$ have been shown to act as weak flux pinning sites, leading to a reversible magnetization¹, magnetization decay² and non-zero resistivity³ in moderate magnetic fields at temperatures near the transition temperature, T_c . These effects limit the technological utility of the as-grown material³. This is not entirely surprising, as both single crystals and polycrystalline materials are grown under conditions expected to minimize the defect density, but the weak pinning in these materials has nevertheless raised the issue of a possible intrinsic limitation to the critical current density. Here we report the attainment of a critical current density, J_c , of $\sim 6 \times 10^5 \text{ A cm}^{-2}$ at 77 K and 9 kOe, in a single crystal of $\text{YBa}_2\text{Cu}_3\text{O}_7$ irradiated with fast neutrons to introduce defects. (In both irradiated and unirradiated crystals, the critical current density was inferred by measuring the magnetic moment resulting from an induced persistent current.) This hundredfold enhancement of J_c relative to unirradiated crystals demonstrates that artificially induced defects can act as strong flux pinning sites, and analysis of the defects should increase our understanding of flux pinning in high- T_c superconductors.

Thin films, in which we expect both different defects and a far higher defect density, can sustain supercurrents of $> 4 \times 10^5 \text{ A cm}^{-2}$ at 77 K and 20 kOe, with low dissipation levels⁴ (measured by magnetization). Until now, such high values have not been reported in bulk $\text{YBa}_2\text{Cu}_3\text{O}_7$. Various methods of artificially increasing the defect density have been tried, including ion irradiation^{5,6} and cation substitution, but the critical current density at 77 K has not been significantly enhanced.

Fast neutron irradiation^{7–11} has also been used, with results ranging from negative to promising. Küpfer *et al.*¹¹, for example, report a twentyfold increase in the intragranular J_c of polycrystalline $\text{YBa}_2\text{Cu}_3\text{O}_7$ at 40 K and 15 kOe. As these ceramics are prepared using a high-temperature sintering step, the intragranular defects, and hence J_c , are similar to those of single crystals¹². Wisniewski *et al.*¹⁰, found a twentyfold increase in the magnetization of ceramic at 77 K after neutron irradiation, but the intragranular J_c cannot be inferred from the reported data. Fleischer *et al.*¹³ found a similar twentyfold increase after irradiation of uranium-doped $\text{YBa}_2\text{Cu}_3\text{O}_7$ with thermal neutrons, and were able to infer critical currents of $\sim 10^5 \text{ A cm}^{-2}$ at 77 K. Previous work on single crystals⁸ has suggested at best a twofold increase in J_c , even though a procedure similar to ours was used.

Our single crystals were prepared as described previously¹⁴. Extensive measurements on these crystals have convinced us that they are uniform, homogeneous, reproducible and generally of high quality. They were encapsulated in silica tubes and irradiated with fast ($E > 1.0 \text{ MeV}$) neutrons at the reactor facility of the University of Virginia, a facility which offers a high degree of shielding from slow neutrons (Cd ratio = 1.0). The neutron fluence was $7.9 \times 10^{16} \text{ cm}^{-2}$.

Figure 1 shows the magnetization of an irradiated crystal during cooling in a 30-Oe applied field ($H \parallel c$ axis). The transition temperature is unchanged from the unirradiated value (89 K) and the sample shows no low-temperature anomalies which might indicate extensive second-phase regions. (The low level of flux expulsion results from the thin-plate geometry.) Zero-

Received 6 June; accepted 20 September 1989.

1. Labitzke, K. & van Loon, H. *J. atmos. terr. Phys.* **50**, 197–206 (1988).
2. van Loon, H. & Labitzke, K. *J. Clim.* **1**, 905–920 (1988).
3. Geller, M. A. *Nature* **332**, 584–585 (1988).
4. Hide, R. & Mason, P. J. *Adv. Phys.* **24**, 47–100 (1975).
5. Shutts, G. J. *Q. Jl R. met. Soc.* **109**, 737–761 (1983).
6. James, I. N. & Gray, L. J. *Q. Jl R. met. Soc.* **112**, 1231–1250 (1986).
7. Wallace, J. M. & Blackmon, M. L. in *Large Scale Dynamical Processes in the Atmosphere* (eds Hoskins, B. J. & Pearce, R. P.) 55–94 (Academic, London, 1983).
8. Chatfield, C. *The Analysis of Time Series: An Introduction* 3rd edn, 142–143 (Chapman and Hall, London, 1984).

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field-cooled measurements show that at low temperatures the sample exhibits essentially complete diamagnetic shielding. These results are consistent with previous studies^{8,9}. Magnetization loops ($H \parallel c$ axis), shown in Fig. 2, were obtained with a vibrating-sample magnetometer. Owing to the low critical current of the unirradiated sample, an ensemble of nominally identical crystals was measured to increase the signal-to-noise ratio. The limited number of samples has allowed us to measure only one irradiated crystal so far, so we cannot report on the reproducibility of the experiment. The magnetization of the irradiated crystal (at $H=0$) did not decay measurably over a time interval of ~ 30 s; this behaviour will be the subject of a further study. A crude estimate of the self-inductance of the sample is ~ 1 nH; thus, the inferred resistivity is in the range $\rho < 10^{-16}$ Ω cm, assuming that the decay is dominated by a simple L/R time constant (where L is inductance and R resistance). The dimensions of the sample ($0.06 \times 0.08 \times 0.003$ cm) preclude a satisfactory estimate of critical-current anisotropy¹⁵, as the broad face is in the a - b plane.

Transport measurements of J_c are both insensitive (to measure 10^{-16} Ω cm at 10^6 A cm⁻² with conventional equipment would require a crystal ~ 1 m long) and necessitate the application of a large current (for a typical cross-section of 0.06×0.003 cm, a current of 240 A would be required). But we can infer the critical current in the plane of the film ($J \parallel a, b$ axes) unambiguously using the critical-state model and a Bean-like interpretation. In this case, $J_c = 20 \Delta M [f(1-f/3g)]^{-1}$ (ref. 15) where ΔM is the difference between the upper and lower magnetization branches at a given field, and f and g ($f < g$) are the face dimensions of the rectangular sample. Here, as throughout the paper, we use the practical units gauss, cm and amperes. At 9 kOe we measure $\Delta M^{\text{irrad}} = 1,200$ G, so $J_c^{\text{irrad}}(77 \text{ K}, 9 \text{ kOe}) = 6.2 \times 10^5$ A cm⁻². A similar analysis of the magnetization of the unirradiated sample yields $J_c^{\text{unirrad}}(77 \text{ K}, 9 \text{ kOe}) = 6.5 \times 10^3$ A cm⁻². Measurements on an unirradiated sample broken from the same original crystal as the irradiated sample were noisy but consistent with the latter value. In these analyses, we assume that the currents flow through the entire sample, that is, that the sample is not granular. To check this, we note that the shielding field H^* required to reverse the direction of the circulating current is given by $H^* \approx 2\pi t J_c / 5$, a value which depends only on the thickness, t , of the sample, and which has been empirically verified on many of our single crystals. The shielding field can be seen to be ~ 3 kOe, so $J_c \approx 8 \times 10^5$ A cm⁻². Unless the induced granularity coincidentally preserves the aspect ratio of the sample, this indicates that the induced currents flow throughout the sample. Note that if

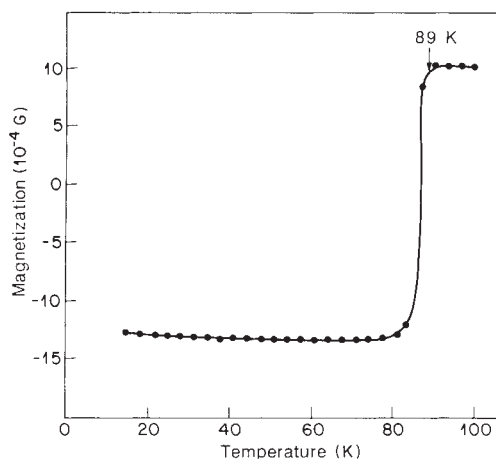


FIG. 1 Temperature dependence of the magnetization for the irradiated sample on cooling in a 30-Oe magnetic field. The absence of structure indicates a relatively homogeneous sample. The offset in magnetization above T_c is due to instrumental background.

the sample were granular, the inferred intragranular critical current would be larger than 6×10^5 A cm⁻² (inversely proportional to the characteristic length of a grain).

We attribute the dramatic increase in J_c on irradiation to the introduction of pinning centres which inhibit flux motion. It is possible that the density of pinning centres has been increased, or that the pinning energy of each centre has increased, or both. Alternatively, the nature of the pinning may have changed, for example, by altering the electronic anisotropy. Note that the anomalous increase in J_c at low fields (> 5 kOe) has been eliminated in the irradiated sample; a similar effect was noted previously in measurements¹¹ at 40 K, but the interpretation remains controversial. In any case, we emphasize that this magnetic measurement probes the sample with extreme sensitivity to dissipation, and that an effective criterion of 10^{-16} Ω cm is sufficiently stringent for almost all technological applications.

The preceding discussion shows that flux creep is certainly not an intrinsic limitation on the use of bulk high- T_c superconductors. Radiation-induced defects are not expected to improve intergranular superconducting connections, as shown by studies on thin films¹⁶, but in bulk materials not dominated by weak-link intergranular connections (for example, materials formed by melt-textured growth¹⁷⁻¹⁹), the artificially induced defects should dramatically enhance the critical current density at 77 K.

The structure of the defects introduced by neutron irradiation has not yet been determined. The defects apparently have a different effect than those produced by heavy-ion irradiation, which have so far given at most a twofold increase⁶ in J_c at 77 K (in thin films). Irradiation by heavy ions creates a high density of defects strongly correlated along the tracks of individual ions. Because neutron collision cross-sections are small, neutron irradiation generates defects much more homogeneously

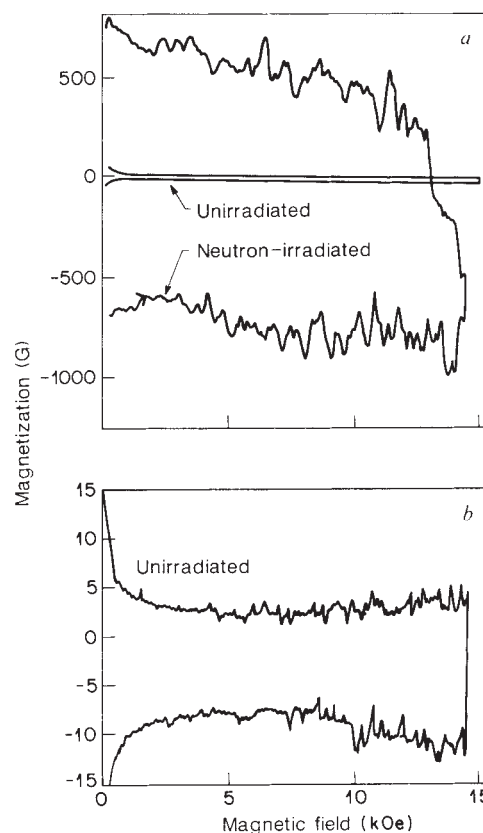


FIG. 2 *a*, Magnetization hysteresis loops of the unirradiated and irradiated sample. The large magnetizations observed in the irradiated sample imply a large circulating supercurrent. *b*, The hysteresis loop of the unirradiated sample on a more sensitive scale. The unirradiated sample is an ensemble of individual crystals, to increase the signal-to-noise ratio.

throughout the volume of our single-crystal samples, and with much lower local defect density. These distinctions may be important, but we also note that nominally identical neutron irradiation procedures have resulted in effects on critical current ranging from moderate enhancement to degradation. □

Received 8 September; accepted 4 October 1989.

1. Müller, K. A., Takashige, M. & Bednorz, J. G. *Phys. Rev. Lett.* **58**, 1143–1145 (1987).
2. Yeshurun, Y. & Malozemoff, A. P. *Phys. Rev. Lett.* **60**, 2202–2205 (1988).
3. Palstra, T. T. M., Battlogg, B., van Dover, R. B., Schneemeyer, L. F. & Waszczak, J. V. *Appl. Phys. Lett.* **54**, 763–765 (1989).
4. Watanabe, K. et al. *Appl. Phys. Lett.* **54**, 575–577 (1989).
5. Valles, J. M. et al. *Phys. Rev. B* **39**, 11599–11602 (1989).
6. Roas, B., Hensel, B., Saemann-Ischenko, G. & Schultz, L. *Appl. Phys. Lett.* **54**, 1051–1053 (1989).
7. Küpfer, H. et al. *Z. Phys.* **69**, 167–171 (1987).
8. Umezawa, A. et al. *Phys. Rev. B* **36**, 7151–7154 (1987).
9. Cost, J. R., Willis, J. O., Thompson, J. D. & Peterson, D. E. *Phys. Rev. B* **37**, 1563–1568 (1988).
10. Wisniewski, A. et al. *Solid St. Commun.* **65**, 577–580 (1988).
11. Küpfer, H. et al. in *The Science of Superconductivity and New Materials* (ed. Nakajima, S.) 172–182 (World Scientific, Singapore, 1989).
12. Jin, S. et al. *Appl. Phys. Lett.* **54**, 584–586 (1989).
13. Fleischner, R. L., Hart, H. R., Lay, K. W. & Luborsky, F. E. *Phys. Rev. B* **40**, 2163–2169 (1989).
14. Schneemeyer, L. F. et al. *Nature* **328**, 601–603 (1987).
15. Gyorgy, E. M., van Dover, R. B., Jackson, K. A., Schneemeyer, L. F. & Waszczak, J. V. *Appl. Phys. Lett.* **55**, 283–285 (1989).
16. White, A. E. et al. *Appl. Phys. Lett.* **53**, 1010–1012 (1988).
17. Jin, S. et al. *Appl. Phys. Lett.* **52**, 2074–2076 (1988).
18. Murakami, M., Morita, M., Doi, K. & Miyamoto, M. *J. appl. Phys.* **28**, 1189–1194 (1989).
19. Salama, K., Selvaranickam, V., Gao, L. & Sun, K. *Appl. Phys. Lett.* **54**, 2352–2354 (1989).

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Water-level fluctuations of Lake Tanganyika in phase with oceanic changes during the last glaciation and deglaciation

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THERE has been considerable controversy about the magnitude of fluctuations of the levels of Lake Tanganyika, the Earth's second deepest lake (1,470 m), following the discovery of submerged valleys extending down to 550 m below present lake levels¹. These fluctuations register changes in the precipitation/(evaporation+evapotranspiration) ratio in a large equatorial-tropical area of catchment, south of the Equator. Here we report new palaeohydrological data, back to 40 kyr BP, from carbon dating of the total organic matter in two diatomaceous cores. The results constrain the vertical lake level fluctuations more than has hitherto been possible^{2–5} and show that the fluctuations from 26 kyr BP are correlated with changes in global sea level and ice volume. Surprisingly fluctuations seem to be in phase with those of the African lakes north of the Equator, which are clearly linked to the Milankovitch mechanisms.

Lake Tanganyika (3°30'–9° S, 31°20' E), a meromictic lake^{6,7}, contains ~18,800 km³ of water⁸. The precipitation on the lake surface and surface runoff represents about 63% and 37% of the water input, respectively; evaporation (94%)^{3,9} is the major loss of water. Surface outflow by the Lukula river controls the maximum (and present) lake level.

Core MPU XII (Figs 1 and 2), 1,013 cm in length, was collected in the southern basin where the lake is 422 m deep, and has been dated to 25,600 BP at its base⁴. Core MPU III (Figs 1 and 3), 1,123 cm in length, was taken at the depth of 130 m on the steep slope that separates the Cameron Bay from the southern basin. The sediments consist mainly of laminated or homogeneous diatomite.

Detailed results of quantitative diatom analyses and taxonomical discussion will be presented elsewhere. Here we focus on the water level indicators (Figs 2 and 3). Diatom biozones, based on the dominant taxa, appear in the cores (Fig. 4). We correlate them with diatom biozones previously established in core T2^{2,3} which was collected close to core MPU XII (Figs 1 and 4). We have also deduced the ages of the Holocene events from core SD2 which was taken from the northern basin (Fig. 4).

We measured the stable isotope (¹³C) contents of total organic matter (TOM) from samples of the core MPU III (Fig. 3) to evaluate the relative proportion of lacustrine and terrestrial organic matter¹⁰. The ¹³C contents are close to -25‰ and the ¹⁴C activity values (from accelerator mass spectrometry, AMS) thus did not require normalization¹¹.

Core MPU XII shows nine diatom biozones distributed in three major stages (Figs 2 and 4). During stage 1 (>25,600–21,700 yr BP), the lake was shallower than today, as indicated by the amount of *Cyclotella ocellata*, a facultative planktonic species commonly regarded as littoral¹². This species predominates in biozones 1A (>25,600–~24,500 yr BP) and 1C (~22,500–21,700 yr BP). The scarcity of periphytic forms (attached on a submerged substrate) is, however, difficult to reconcile with very shallow environments, or with a massive transport of diatoms from the marginal zone. Modern analogues are not available for assemblages rich in *Cyclotella ocellata* in recent African lakes¹³ although this species was common in the plankton of Ethiopian and Afar lakes during the late Pleistocene¹⁴, such as Lake Abhé which was then 100–160 m deep¹⁵. Such a moderate depth at the MPU XII site would be compatible with both the low percentages of periphytic forms and with the absence of lamination which suggests that the top of the anoxic deep-water mass, presently lying at a depth of 100–150 m (ref. 6), was below the core site during the sedimentation of biozones 1A and 1C. In contrast, the laminated facies in biozone 1B indicates a permanent water stratification. The relatively high proportion of planktonic forms and the permanency of *C. ocellata* are further indications of a moderate rise in water level centred around 24,000 yr BP.

Stage II (21,700–12,700 yr BP) corresponds to generally very shallow conditions. Periphytic diatoms (such as *Rhopalodia* spp.) became abundant. They are associated with sponge spicules and phytoliths which also reflect shallow environments. Few laminations are observed. The minimum water depth is recorded in biozone IIB by a peak of *Gomphonema clevei*, typically periphytic in modern East Africa¹³. The presence of intact frustules (connected valves) is a further indication that this species was growing *in situ* at the MPU XII site, which thus was located in the photic zone. This reveals a drop in water

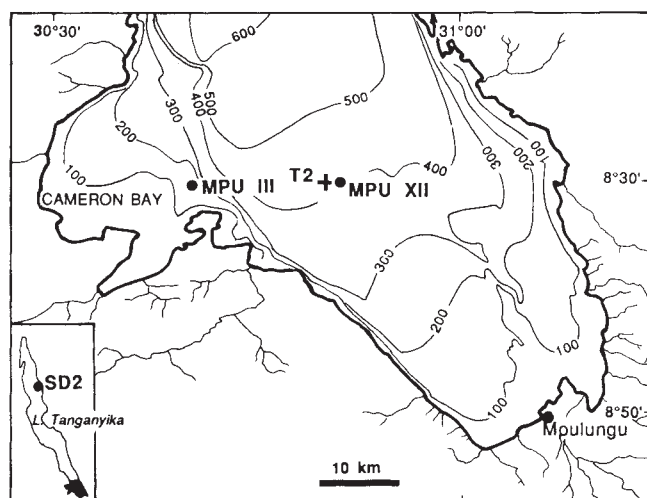


FIG. 1 Core location in Lake Tanganyika.

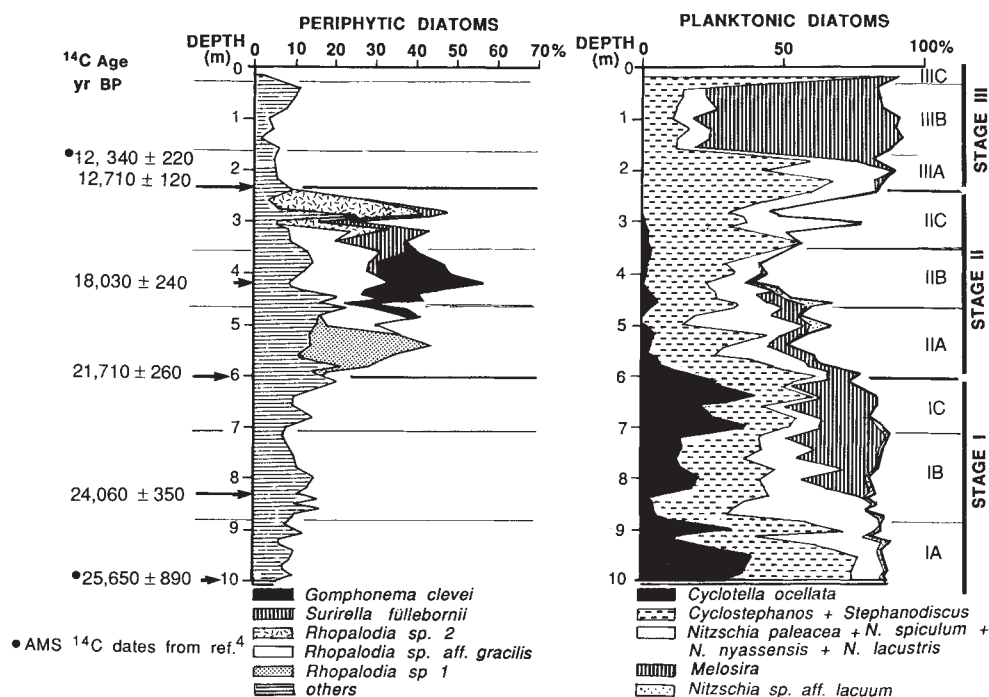


FIG. 2 Distribution of periphytic and planktonic diatoms along core MPU XII.

level of at least 350 m, more probably of about 400 m; this corresponds to a water volume that is -40 to -60% of the present value. The lake area/drainage area ratio was only 0.090-0.070 at that time compared to a present value of 0.137. The *Gomphonema* peak is dated at $18,030 \pm 240$ yr BP. This date differs significantly from that previously inferred from the ^{14}C -dated secular palaeomagnetic variations obtained in a Cameroon lake and extrapolated to Lake Tanganyika⁴. Whatever the causes for this discrepancy (for example, limited resolution of conventional ^{14}C ages from the Cameroon materials or delayed magnetic reorientation of fine particles in the highly porous sediments from Lake Tanganyika), such extrapolations seem to be unwarranted.

A generally positive lake water balance was established during Stage IIC ($\sim 16,000$ ->12,700 yr BP), and the planktonic flora indicates that deep conditions were definitely restored to the lake shortly before 12,700 yr BP. This event was possibly preceded by a first-positive oscillation around 14,500 yr BP, as suggested by a narrow peak of planktonic forms observed at a

depth of 300 cm (Fig. 2). Deep-water conditions were maintained during Stage III, from 12,700 yr BP onwards. Perennial stratification occurred from 12,700-~10,600 yr BP (base of biozone IIIA; Fig. 4) and from ~5000 yr BP, whereas biozone IIIB indicates deep mixing which may be due to upwelling events⁵.

In core MPU III (Fig. 3), detritus deposits and disturbances are observed in the intervals 981-935 cm and 880-780 cm, and we therefore do not consider them in our palaeohydrological reconstructions. A discontinuity is suggested by the ^{14}C dates between ~30,000 and >12,300 yr BP, which may explain the absence of stages I and IIA-B.

Core MPU III confirms that the major regression ends around 13,000 yr BP. We assign the core section 780-760 cm to biozone IIC, and deep-water conditions were established before 12,300 yr BP (biozone IIIA) and maintained to the present. From >12,300-10,600 BP, a progressive decrease in the ^{13}C values (Fig. 3) may reflect the establishment of an arboreal vegetal cover on the catchment area⁴, indicating increasing temperature and humidity¹⁶. The marked decrease at ~10,600 BP indicates

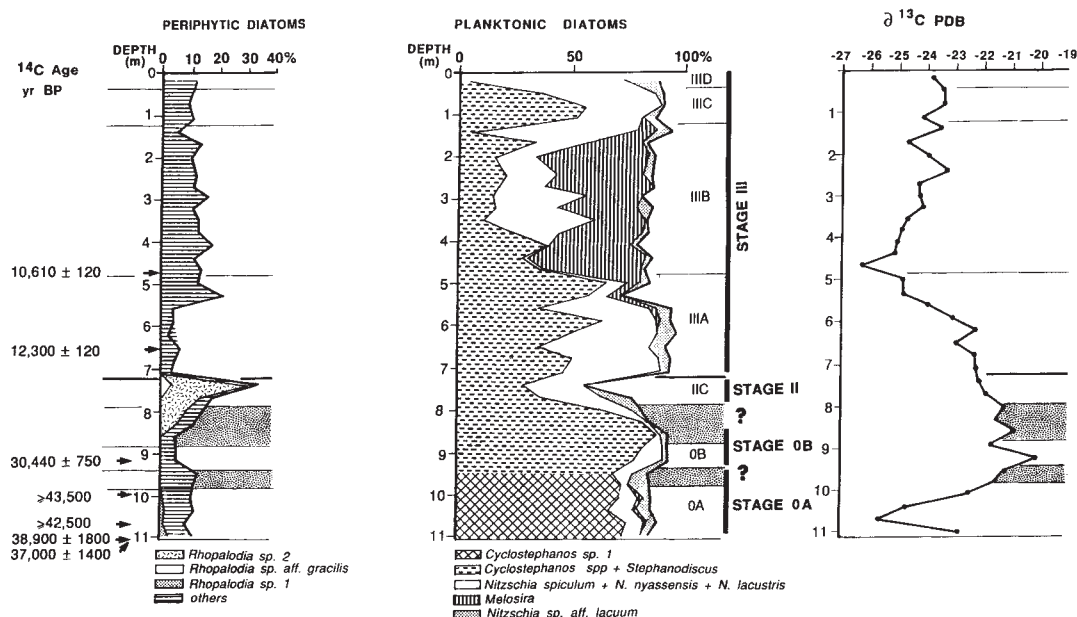
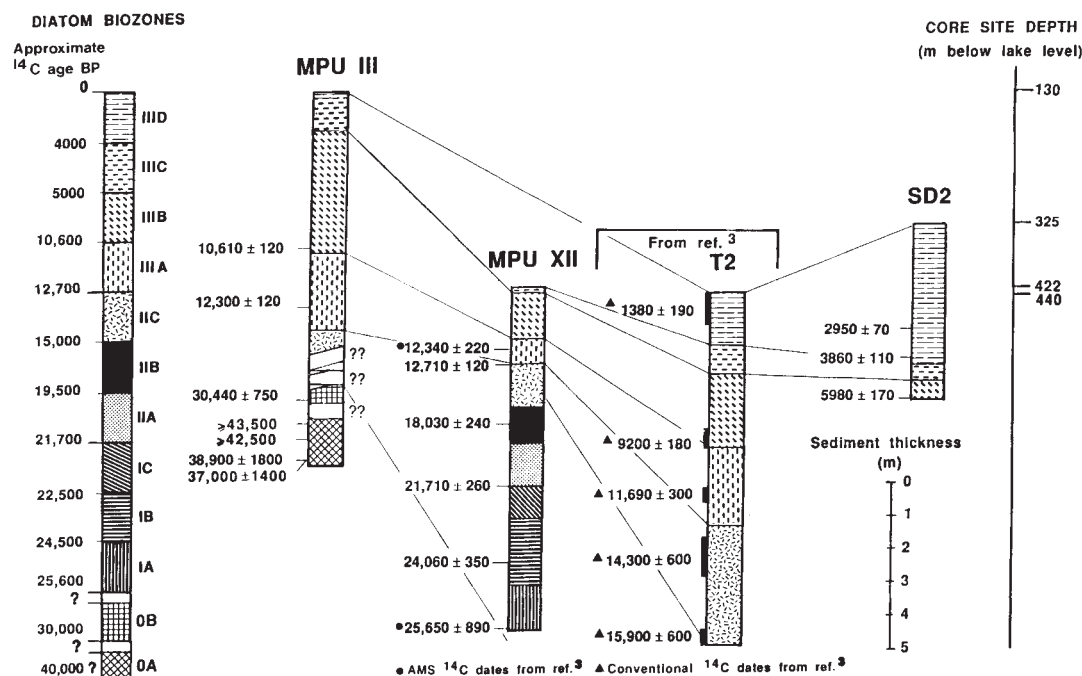


FIG. 3 Distribution of periphytic and planktonic diatoms, and ^{13}C content of total organic matter along core MPU III.

FIG. 4 Tentative correlations between four cores from Lake Tanganyika using diatom biozones. Core T2 from ref. 3. Diatom biozones, abundant taxa: OA, *Cyclotellina* sp. 1; OB, *Cyclotellina damasii*, needle-like *Nitzschia* (*N. spiculum*, *N. nyassensis*, *N. lacustris*; IA: *Cyclotellina ocellata*, needle-like *Nitzschia*; IB: *Cyclotellina* spp. + *Stephanodiscus* spp. needle-like *Nitzschia*, *Cyclotellina ocellata*; IC: *Cyclotellina ocellata*, *Melosira* spp.; IIA: *Rhopalodia* sp. 1., *Cyclotellina* spp. + *Stephanodiscus* spp.; IIB: *Gomphonema clevei*, *Rhopalodia* sp. aff. *gracilis*; IIC: *Cyclotellina* spp. + *Stephanodiscus*, *Surreirella fullebornii*, *Rhopalodia* spp.; 2; IIIA: *Cyclotellina* spp. + *Stephanodiscus* spp., needle-like *Nitzschia*; IIIB: *Melosira* spp.; IIIC: *Cyclotellina damasii*; IIID: needle-like *Nitzschia*. (needle-like is the terminology used in ref. 3).



the maximum input of organic matter of terrestrial origin and an abrupt increase in rainfall and related runoff which coincides with the limit between biozone IIIA and IIIB.

Below 920 cm, the diatom flora reveals the occurrence of two Late Pleistocene stages of high water level (Fig. 3). The youngest, stage OB, is dated to $30,440 \pm 750$ yr BP. Facies and diatom flora resemble that of biozone IIIA. The ^{13}C content reflects an almost exclusive aquatic origin of the organic matter. The oldest, stage OA, is characterized by a planktonic diatom assemblage unknown in core MPU XII. Its age falls at or close to the limit of the ^{14}C method (Fig. 3). The slight discrepancies observed between the three measured ages may be due to the presence of detrital organic matter, because the low ^{13}C values indicate an important input of terrestrial material. Such a supply is indistinguishable in the core stratigraphy. An alternative explanation may thus be found in a slight contamination of the 1,108-cm sample which repeatedly provided a still measurable ^{14}C age (Fig. 3).

This survey shows that lake level fluctuations from 26,000 yr BP correlate with changes in global sea level and ice volume¹⁷: the lake level is intermediate from 26,000 to 21,700 yr BP, is low from 21,700 to 13,000 yr BP with a minimum at 18,000 yr BP and is high from 13,000 yr BP onwards. The reestablishment of a positive water balance occurred during the first step of the deglaciation, but the refilling of the lake is not linear. The abruptness of the major hydrological events recorded at $\sim 13,000$ yr BP and $\sim 10,600$ yr BP makes the fluctuations of the lake water balance synchronous with the deglaciation steps recorded in the Atlantic ocean^{18,19}. Lake Tanganyika fluctuations seem to be in phase with those of the African lakes north of the Equator²⁰ which are clearly linked to the Milankovitch mechanisms²¹. From the general circulation models we might have expected opposite phases as a result of different insolation values from the astronomical theory of palaeoclimates²². Conditions wetter than the present ones, which are predicted by a simulation²³ at 18,000 yr BP in East Africa and attributed to warmer surface temperature of the equatorial Indian Ocean, are not confirmed. More simply, the water balance of Lake Tanganyika reflects the hydrological ocean variations that are

due to glaciation/deglaciation processes and the related greater availability of global atmospheric moisture during warmer global climatic phases.

Note added in proof: We have become aware of a related paper²⁴ which considers the possibility of a time lag of some centuries in the accumulation of organic matter. This hypothesis is based on the isotope balance of C_4/C_3 plants as an index of *in situ* (*Cyperaceae*) versus reworked (trees) organic matter. However, the planktonic contribution, with ^{13}C content lower than for *Cyperaceae*, is neglected, and thus the time-lag estimate is pessimistic.

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- Capart, A. *Exploration hydrobiologique du Lac Tanganyika (1946-47)*, vol. 2, 1-16 (Inst. R. Sci. Nat. Belg., Bruxelles, 1949).
- Livingstone, D. A. *Limnol. Oceanogr.* **10**, 607-610 (1965).
- Haberyan, K. A. & Hecky, R. E. *Palaeogeogr. Palaeoclimatol. Palaeoecol.* **61**, 169-197 (1987).
- Tiercelin, J. J. et al. *C. R. hebdo. Séanc. Acad. Sci., Paris* **307**, 1375-1382 (1988).
- Scholz, C. A. & Rosendahl, C. A. *Science* **240**, 1645-1648 (1988).
- Degens, E. T., Van Herzen, R. P. & Wong, H. K. *Naturwissenschaften* **58**, 229-251 (1971).
- Stoffers, P. & Hecky, R. E. in *Int. Ass. Sedimentol., Spec. pub. 2*, 43-55 (1978).
- Hecky, R. E. & Degens, E. T. *Woods Hole Oceanographic Institution, Tech. Rep.* 73-78 (1978).
- Beadle, L. C. *The Inland Waters of Tropical Africa* (Longman, London, 1981).
- Deines, P. in *Handbook of Environmental Isotope Geochemistry* Vol. 1 (eds Fritz, P. & Fontes, J. Ch.) 329-406 (Elsevier, Amsterdam, 1980).
- Stuiver, M. & Polach, H. A. *Radiocarbon* **19**, 355-363 (1977).
- Hustedt, F. in *Das Phytoplankton des Süßwassers* Vol. 16 (ed. Thiennemann, A.) (Schweizerbart, Stuttgart, 1942).
- Gasse, F. in *Bibliotheca Diatomologica* Vol. 11 (Cramer, Stuttgart, 1986).
- Gasse, F. & Street, F. A. *Palaeogeogr. Palaeoclimatol. Palaeoecol.* **24**, 279-325 (1978).
- Gasse, F. *Nature* **242**, 42-45 (1977).
- Vincens, A. *Palaeobotany and Palynology* (Elsevier, Amsterdam, in the press).
- Labeyrie, L. D., Duplessy, J. C. & Blanc, P. L. *Nature* **367**, 477-482 (1987).
- Bard, E. et al. *Science* **228**, 791-794 (1987).
- Berger, W. H., Killingley, J. S. & Vincent, E. *Nature* **314**, 156-158 (1985).
- Street-Perrott, F. A. & Roberts, N. in *Variations in the Global Water Budget* (eds Street-Perrott, F. A. et al.) 331-445 (Reidel, Dordrecht, 1983).
- Kutzbach, J. E. & Street-Perrott, F. A. *Nature* **317**, 2130-2134 (1985).
- Berger, A. *J. Atmos. Sci.* **35**, 2362-2367 (1978).
- COHMAP Members. *Science*, **241**, 1043-1052 (1988).
- Hillaire-Marcel, C. et al. *Quat. Sci. Rev.* **8**, 206-211 (1989).

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Glacial-interglacial climatically forced $\delta^{13}\text{C}$ variations in sedimentary organic matter

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SEDIMENTARY organic geochemical properties have long been used to characterize sedimentary organic matter sources¹⁻⁸, but the characterizations are often not unique. There are at least four plausible mechanisms to account for the large (~3-6‰) glacial-to-interglacial climatic variations in the sedimentary organic-carbon isotopic composition ($\delta^{13}\text{C}_{\text{org}}$) in the northern Gulf of Mexico. They are: temperature-dependent C isotopic fractionation by phytoplankton, mixing of C_3 -photosynthetic terrigenous and marine organic matter, mixing of C_3 - and C_4 -photosynthetic organic matter and diagenetic alteration of the originally sedimented material. In a 208.7-m Deep Sea Drilling Project hydraulic-piston core (DSDP 619) from the Pigmy Basin in the northern Gulf of Mexico, we find that paired analyses of $\delta^{13}\text{C}_{\text{org}}$ and N/C are consistent with the hypothesis that the sedimentary organic carbon in the Pigmy Basin is a glacial-interglacial climatically determined mixture of C_3 -photosynthetic terrigenous and marine organic matter⁹, confirming the model of Sackett⁵.

The Pigmy Basin is a late Quaternary blocked-canyon intra-slope basin the northern Gulf of Mexico¹⁰ (Fig. 1). The basin has normally oxic and saline bottom waters¹¹. In interglacial times (isotope (sub)stages 1 and 5a,b) sedimentation rates ranged from 60 to 85 cm kyr^{-1} , whereas during glacial times (isotope stages 2 and 4) sedimentation rates varied from 230 to 250 cm kyr^{-1} as determined from the DSDP Hole 619 hydraulic piston core^{9,12}. The occurrence of higher sedimentation rates in glacial stages was presumably due to increased erosion of continental material during falling sea levels and low sea-level stands¹³.

We present the sedimentary organic-carbon isotopic composition ($\delta^{13}\text{C}_{\text{org}}$) of 55 samples from the Pigmy Basin as a time series (Fig. 2a). The carbon isotope results are given in the δ -notation relative to the Chicago PDB standard, $\delta^{13}\text{C}_{\text{org}} = (R_{\text{S}}/R_{\text{R}} - 1) \times 1,000$ where R_{S} and R_{R} are the $^{13}\text{C}/^{12}\text{C}$ ratios of sample and reference CO_2 gases⁴. The interglacial oxygen-isotope stages 1 and 5a,b are characterized by relatively positive average ($\pm 1\sigma$) $\delta^{13}\text{C}_{\text{org}}$ values (stage 1: $-24.2 \pm 1.2\text{‰}$, $n = 9$; sub-stages 5a,b: $-23.0 \pm 0.8\text{‰}$, $n = 7$), whereas stages 2 and 3 have comparatively negative average values (stage 2: $-25.6 \pm 0.5\text{‰}$, $n = 13$; stage 3: $-25.2 \pm 1.3\text{‰}$, $n = 18$). Isotope stage 4 has two relatively negative values (average -25.3‰) in the middle of the stage with relatively positive values near the stage 4 boundaries. (3/4 boundary: -22.8‰ ; 4/5 boundary -21.9‰). To our knowledge this time series is the highest-resolution record of $\delta^{13}\text{C}_{\text{org}}$ in the Gulf of Mexico spanning from the present interglacial isotope stage 1 to the previous interglacial isotope substage 5b (~96 kyr BP).

Sackett observed a 4.8‰ increase in the carbon isotopic composition of sedimentary organic matter over the last ~20 kyr on the Sigsbee Knolls (core V3-127) in the northern Gulf of Mexico⁵. He attributed the temporal $\delta^{13}\text{C}_{\text{org}}$ increase to mixing of terrigenous and marine organic carbon sources by analogy with the apparent spatial mixing of terrigenous and marine organic carbon in Recent eastern Gulf Coast surface sediments.

The temporal variation of $\delta^{13}\text{C}_{\text{org}}$ is consistent with a glacial-to-interglacial climatically forced mixing of terrigenous and marine organic carbon⁵ and the effects of the ~100-kyr cycle of climatic change¹⁴.

Rogers and Koons⁶ examined the $\delta^{13}\text{C}_{\text{org}}$ records of 24 gravity and piston cores and four 330-m drill cores in the northern Gulf of Mexico, with the longest record extending into the upper Pliocene (~2 Myr). They found that warm stages (as defined by planktonic foraminiferal assemblages) were characterized by $\delta^{13}\text{C}_{\text{org}}$ values of -20 to -22‰ (relative to PDB), whereas cold stages had $\delta^{13}\text{C}_{\text{org}}$ values of -24 to -26‰. They eliminated sedimentary diagenesis of organic carbon as a cause for $\delta^{13}\text{C}_{\text{org}}$ variations because over the time spanned (~2 Myr) there was no secular monotonic trend in $\delta^{13}\text{C}_{\text{org}}$ values. They concluded that the major cause of the $\delta^{13}\text{C}_{\text{org}}$ variations was the temperature-dependent fractionation of carbon isotopes by phytoplankton, and considered mixing of terrigenous and marine organic carbon an unlikely cause.

Given the sedimentary $\delta^{13}\text{C}_{\text{org}}$ variations in the northern Gulf of Mexico, Sackett and Rankin⁷ showed that the glacial-to-interglacial sea surface temperature variation predicted from the temperature-induced fractionation of carbon isotopes (35 °C) would be implausibly large in light of the oxygen isotopic composition of contemporaneous planktonic foraminifera¹⁵. As a temperature change of only 6-10 °C from the last glacial to the present interglacial had been calculated earlier with the $\delta^{18}\text{O}$ of planktonic foraminifera in Caribbean surface waters¹⁵, they favoured the variation of relative proportions of terrigenous and marine organic carbon inputs as the cause of the temporal $\delta^{13}\text{C}_{\text{org}}$ changes. Later, the applications of a foraminiferal-assemblage temperature-transfer method^{16,17} and the foraminiferal oxygen-isotope temperature method using revised ice volume estimates^{9,18} indicated only small (0.8-2 °C) late Quaternary temperature variations in the northern Gulf of Mexico. These results make temperature-dependent carbon

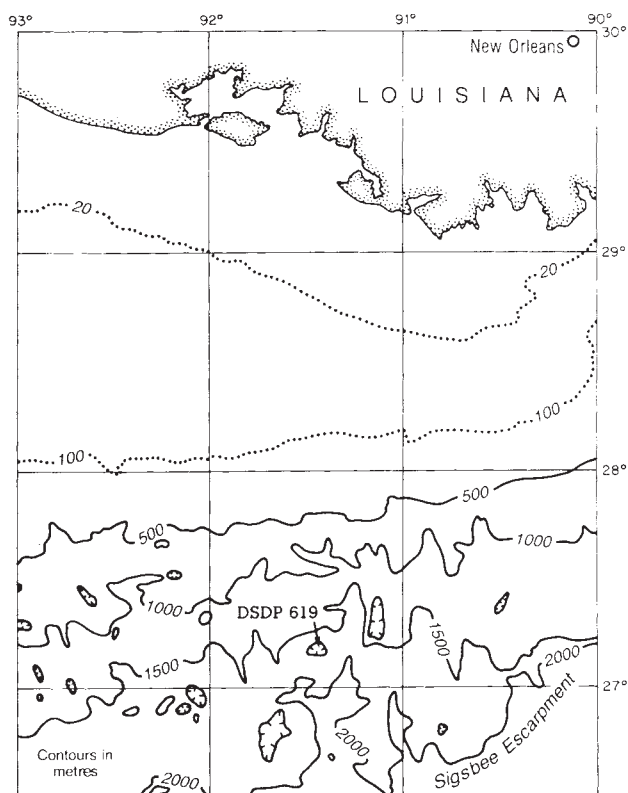


FIG. 1 Map showing cored site on Deep Sea Drilling Project Leg 96, including the Pigmy Basin (Site 619) at 27°11.61' N, 91°24.54' W.

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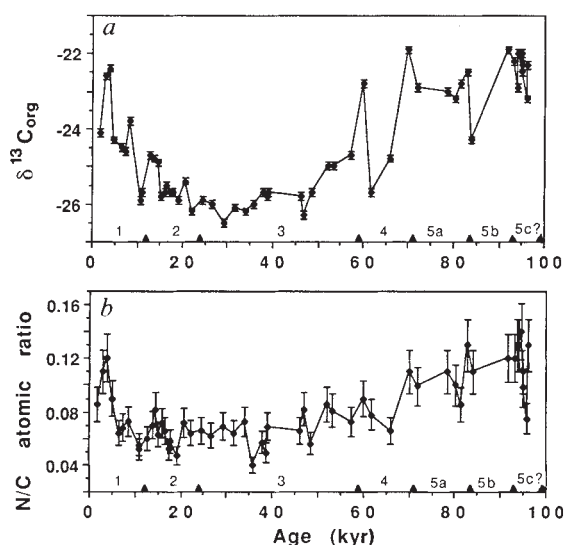


FIG. 2 *a*, Time series of the sedimentary organic-carbon isotopic composition ($\delta^{13}\text{C}_{\text{org}}$) of DSDP 619 in the Pigmy Basin, showing relatively positive (marine) values in interglacial stages and relatively negative (terrigenous) values in glacial stages. *b*, Time series of N/C atomic ratios in DSDP 619 shows the general interglacial predominance of marine organic matter (typically, N/C = 0.13–0.20) and the glacial predominance of terrigenous organic matter (typically, N/C = 0.01–0.05).

isotopic fractionation an unlikely explanation for the large $\delta^{13}\text{C}_{\text{org}}$ variations observed in the DSDP 619 core.

Neuman *et al.*⁸ observed that the $\delta^{13}\text{C}_{\text{org}}$ values of “olive grey, Pleistocene turbidite muds” were -22 to -24% (relative to PDB) and that the $\delta^{13}\text{C}_{\text{org}}$ values for “orange and yellow, Holocene and interglacial foraminiferal oozes” were -20 to -16% . Analysis of the $\delta^{13}\text{C}_{\text{org}}$ records in 48 piston cores in all the major deep-water provinces of the late Quaternary Gulf of Mexico led them to conclude that the temporal $\delta^{13}\text{C}_{\text{org}}$ variations were consistent with increased proportions of terrigenous organic-carbon deposition relative to marine organic-carbon deposition in glacial stages over broad expanses of the Gulf’s sea floor.

The final alternative is that the sediments of the northern Gulf of Mexico contain a climatically determined mixture of C_3 - and C_4 -photosynthetic organic matter. Terrigenous C_3 -photosynthetic organic matter could be eroded into the Gulf from terrigenous sources in glacial times. In interglacial times, C_4 -organic matter could be contributed from C_4 -photosynthetic coastal plants¹⁹ or other ^{13}C -rich sea grasses²⁰ into the deep Gulf of Mexico. We consider this possibility below.

The N/C ratios of DSDP 619 sediments are presented as a time series (Fig. 2*b*). The interglacial (sub)stages 1 and 5a–b are characterized by relatively high N/C values (stage 1: 0.08 ± 0.02 ($\bar{X} \pm 1\sigma$), $n = 9$; substages 5a,b: 0.11 ± 0.02 , $n = 7$). These N/C

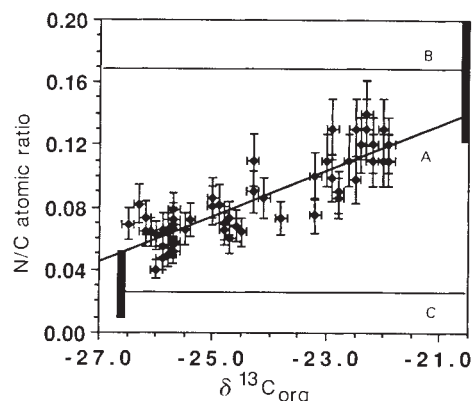
values approach those of planktonic organic matter¹ (0.13–0.20). The average N/C value of glacial stages 2–4 is 0.06 ± 0.02 which overlaps with the range of typical terrigenous plant and peat values of 0.01–0.05 (refs 2, 3). The N/C time series for the Pigmy Basin is consistent with the model of climatically forced glacial-to-interglacial terrigenous- and marine-organic-carbon mixing, reflecting the general ~ 100 kyr climatic cycle.

In Fig. 3 we plot 55 $\delta^{13}\text{C}_{\text{org}}$ values against their corresponding N/C values from the Pigmy Basin core. A linear least-squares regression of N/C on $\delta^{13}\text{C}_{\text{org}}$ shows that the measurements are well correlated ($r^2 = 0.74$, $P < 0.0001$) according to the statistical *F*-test for significance²¹. The correlation of N/C and $\delta^{13}\text{C}_{\text{org}}$ values for the sedimentary organic matter in the Pigmy Basin is consistent with a mixing of C_3 -photosynthetic (Calvin-Benson Cycle of carbon fixation) terrigenous and marine organic matter sources. A linear regression line (labelled A) through the paired data extrapolates through typical terrigenous (0.01–0.05) and marine (0.13–0.20) N/C values at empirically determined $\delta^{13}\text{C}_{\text{org}}$ terrigenous (-26.6% , relative to PDB) and marine (-20.6%) end-member values⁹.

These data support the climatically driven terrigenous- and marine-organic-carbon mixture model and are evidence against other hypotheses for organic-matter deposition in the late Quaternary Gulf of Mexico. If temperature-dependent fractionation of carbon isotopes by marine phytoplankton determined the Quaternary sedimentary $\delta^{13}\text{C}_{\text{org}}$ values, we would expect constant marine N/C values (0.13–0.20) to co-occur with variable $\delta^{13}\text{C}_{\text{org}}$ values (line B) contrary to observation. This observation is consistent with the assertion that the maximum Quaternary surface-water temperature variation in the Gulf of Mexico would cause only an $\sim 1\%$ difference in $\delta^{13}\text{C}_{\text{org}}$, if it had any effect at all⁸. If a climatically determined mixture of C_3 - and C_4 - terrigenous organic matter caused the temporal $\delta^{13}\text{C}_{\text{org}}$ variation, we would observe relatively constant terrigenous N/C values (0.01–0.05) across the range of $\delta^{13}\text{C}_{\text{org}}$ values (line C). This is also contrary to observation.

Sedimentary organic-carbon isotope results from coastal marshes to Recent deep oceanic environments, showed that there was no convincing evidence for significant diagenetic alteration of the sedimentary $\delta^{13}\text{C}_{\text{org}}$ records²². Although the possibility of diagenetic alteration of Quaternary $\delta^{13}\text{C}_{\text{org}}$ records has been discussed⁶, we are unaware of results that support sedimentary diagenetic carbon isotopic alteration as the cause of large $\delta^{13}\text{C}_{\text{org}}$ variations (3–6‰) as those observed in the Quaternary Gulf of Mexico sediments^{5–9}. Rogers and Koons⁶ considered carbon isotopic fractionation during diagenesis to be an insignificant problem affecting the $\delta^{13}\text{C}_{\text{org}}$ record of 0–2-Myr-old sediments because they did not observe a secular trend in the $\delta^{13}\text{C}_{\text{org}}$ values. A later study of the $\delta^{13}\text{C}_{\text{org}}$ of the algal sapropelic sediments of the alternately aquatic, then marine Mangrove Lake in Bermuda, however, indicated an overall decrease in $\delta^{13}\text{C}_{\text{org}}$ of up to $\sim 4\%$ over the past 5 kyr (ref. 23). Therefore, $\delta^{13}\text{C}_{\text{org}}$ records should not be interpreted solely in

FIG. 3 This plot of N/C against $\delta^{13}\text{C}_{\text{org}}$ shows that the composition of sedimentary organic matter in the Pigmy Basin (DSDP 619) for the last ~ 100 kyr is consistent with mixing of terrigenous and marine C_3 -photosynthetic (Calvin-Benson Cycle) organic matter. The linear least-squares regression line (A) is: $\text{N/C} = 0.015 \cdot (\delta^{13}\text{C}_{\text{org}}) + 0.44$ ($P < 0.0001$, $r^2 = 0.74$, $n = 55$). For comparison, typical N/C ratios of terrigenous (0.01–0.05) and marine (0.13–0.20) plants are given at $\delta^{13}\text{C}_{\text{org}}$ terrigenous (-26.6%) and marine (-20.6%) end-member values⁹. Constant marine N/C values with variable $\delta^{13}\text{C}_{\text{org}}$ values (line B) would support temperature-dependent carbon isotopic fractionation as the cause of the late Quaternary Gulf of Mexico $\delta^{13}\text{C}_{\text{org}}$ variations. Constant terrigenous N/C ratios for all $\delta^{13}\text{C}_{\text{org}}$ values would support the mixing of C_3 - and C_4 -terrigenous plant material as the cause of the $\delta^{13}\text{C}_{\text{org}}$ variations (line C). Neither of the latter two hypotheses (represented by lines B and C) are supported by these data.



terms of organic matter sources, particularly in organic-rich sapropels. In the Mangrove Lake, secularly increasing $\delta^{13}\text{C}_{\text{org}}$ values were the result of preferential degradation of selected organic-matter components²³. This mechanism, however, cannot explain the glacial-interglacial cyclic co-variation in $\delta^{13}\text{C}_{\text{org}}$ and N/C values in the Pigmy Basin. Changes in C_3 -photosynthetic terrigenous and marine organic matter sources are supported as the principal source ($\sim 74\%$) of the variation of the $\delta^{13}\text{C}_{\text{org}}$ and N/C records, whereas sedimentary carbon isotopic fraction-

ation, temperature effects on phytoplanktonic carbon isotopic fractionation and mixing of C_3 - and C_4 -photosynthetic organic matter may account for some fraction of remaining variation ($\sim 26\%$). These organic-matter source changes are internally consistent with the inverse relations of selected marine (C_{37} – C_{39} alkenones) and terrigenous (C_{27} – C_{29} desmethyl sterols) organic biomarker compounds to $\delta^{13}\text{C}$ values also found in the Pigmy Basin sediment core (ref. 9; J.P.J. and R.B.G., manuscript in preparation). □

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1. Redfield, A. C., Ketchum, B. H. & Richards, F. A. in *The Sea*, Vol. 2 (ed. Hill, M. N.) 26–77 (Wiley, New York, 1962).
2. Waksman, S. A. *Soil Sci.* **36**, 125–147 (1933).
3. Brenner, S., Ikan, R., Agron, N. A. & Nissebaum, A. *Soil Sci.* **125**, 226–232 (1978).
4. Craig, H. *Geochim. cosmochim. Acta* **3**, 53–92 (1953).
5. Sackett, W. M. *Mar. Geol.* **2**, 173–185 (1964).
6. Rogers, M. A. & Koons, C. B. *Trans. Gulf Coast Ass. geol. Soc.* **19**, 529–534 (1969).
7. Sackett, W. M. & Rankin, J. G. *J. geophys. Res.* **75**, 4557–4560 (1970).
8. Neuman, J. W., Parker, P. L. & Behrens, E. W. *Geochim. cosmochim. Acta* **37**, 225–238 (1973).
9. Jasper, J. P. thesis, Mass. Inst. Tech./Woods Hole Oceanographic Inst., WHOI-88-28 (1988).
10. Bouma, A. H. *Mem. Am. Ass. Petrol. Geol.* **34**, 567–581 (1983).
11. Bouma, A. H., Stelling, C. E. & Leg 96 Sedimentologists *Init. Rep. DSDP* **96**, 563–576 (1986).
12. Williams, D. F. & Kohl, B. *Init. Rep. DSDP* **96**, 671–676 (1986).
13. Broecker, W. S. *Geochim. cosmochim. Acta* **46**, 1689–1705 (1982).
14. Broecker, W. S. & van Donk, J. *Rev. Geophys. Space Phys.* **8**, 169–198 (1970).

15. Emiliani, C. *J. Geol.* **63**, 538–578 (1955).
16. Brunner, C. A. *Quat. Res.* **17**, 105–119 (1982).
17. Brunner, C. A. & Cooley, J. F. *Geol. Soc. Am. Bull.* **87**, 681–686 (1976).
18. Jasper, J. P. & Gagosian, R. B. *Palaeoceanography* (in the press).
19. Fry, B., Jeng, W.-L., Scalan, R. S. & Parker, P. L. *Geochim. cosmochim. Acta* **42**, 1299–1302 (1978).
20. Roy Benedict, C., Wong, W. L. & Wong, J. H. H. *Pl. Physiol.* **35**, 512–517 (1980).
21. Snedecor, G. W. & Cochran, W. G. *Statistical Methods* (Iowa State University Press, 1978).
22. Delnes, P. in *Handbook of Environmental Isotope Geochemistry* Vol. 1 (eds Fritz, P. & Fontes, J.) (Elsevier, Amsterdam, 1980).
23. Spiker, E. C. & Hatcher, P. G. *Org. Geochem.* **5**, 283–290 (1984).

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Sr isotope constraints on the Mediterranean environment at the end of the Messinian salinity crisis

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PROBABLY the most profound event that has affected the Mediterranean is the desiccation which occurred at the end of the Miocene^{1–8}. During this period, there was a very rapid change from deep, open-marine conditions to environments of shallow-water carbonate and massive evaporite deposition^{1–10}. Here we show that ostracod valves found in Messinian Lago Mare (Lake Sea) sediments, have a uniform but generally lower Sr isotopic composition than contemporary sea water thus providing evidence that some of the major Mediterranean basins (for example, Balearic and Levantine basins) formed large lacustrine systems that were probably interconnected towards the end of the Messinian salinity crisis^{1–3} after the major evaporites were deposited. The most likely source of fresh water is from continental rivers flowing from the eastern European inland Paratethys sea or perhaps the Nile River. In the lower part of the Lago Mare, some ostracods have Sr isotopic compositions identical to contemporary sea water indicating a transient influence of marine-derived water. Sr compositions greater than that in contemporary sea water are only found in ostracods from onshore sediments in the Sorbas Basin^{4,5} of southeastern Spain suggesting that this small basin, adjacent to the Straits of Gibraltar, became isolated after the deposition of the major evaporite sequences.

The events leading up to and during the so-called 'Messinian salinity crisis' have been the subject of diverse interpretations^{6–11}. Here we discuss whether the various basins in the Mediterranean were interconnected during the Messinian, the source and quantity of continental drainage flowing into the Mediterranean during that period, whether the Paratethys connection was still open, and whether marine water inundated the

Mediterranean at the end of the Messinian salinity crisis, when the Mediterranean consisted of a series of small lakes or a large lake, referred to as Lago Mare^{6,8,11}.

We assume¹² that the Sr isotopic composition of water masses isolated from the open oceans will, in part, reflect the composition of the continental drainages flowing into the closed basin. As the Sr isotopic composition of sea water is now precisely defined throughout the Tertiary^{13–16} it is now possible to resolve even relatively small differences (parts per 10⁵) from open-marine environments. In addition, the Sr isotopic composition in all present-day oceans (including the Mediterranean) are identical within analytical errors¹³. With the collision of Eurasia and Africa¹⁷ and the progressive closure of the Tethys Ocean, the Mediterranean became separated from the eastern inland sea, Paratethys, which had previously provided an Indo-Pacific oceanic connection. The Mediterranean's last links to the open-marine environment were probably through the Betic and Rif Straits on the western side (adjacent to the present-day Straits of Gibraltar) which closed in the middle-late Miocene¹⁸. Thus, with its total isolation, together with the general trend to drier and cooler climate, the Mediterranean was destined for a 'salinity crisis'¹. We expect this to be reflected in a change of the Sr isotopic composition in the Mediterranean lacustrine system relative to that of the open-marine Messinian sea water.

Ostracods are ubiquitous bivalved microcrustaceans that can live in continental, estuarine, marine and hypersaline waters¹⁹. Unlike molluscs and foraminifers, which continue to secrete additional layers or chambers, ostracods are arthropods and have to moult and build new valves after shedding the old ones²⁰. The components of their low-Mg calcite shells are taken directly from the host water after moulting^{21,22} and hence the Sr isotopic composition of the valves is an accurate monitor of the ambient composition of the aquatic environment. The particular ostracod we chose belongs to the well-known euryhaline genus *Cyprideis*. It is cosmopolitan and tolerates a wide range of salinities; modern representative of this genus can be found in fresh waters and in waters with salinities up to 100‰²³. We analysed ostracods from several Deep-Sea Drilling Program (DSDP) sites (129A, 372 and 376) as well as two onshore sites in Spain and Cyprus. All samples are of Messinian age and postdate the evaporitic depositional phase of gypsum and/or halite, and thus date from very close to the Miocene/Pliocene boundary. We determined the Sr isotopic composition of carefully selected, unaltered single ostracod valves following the procedures outlined in ref. 12. A systematic study of the Sr/Ca ratios in the valves

(Table 1) indicated that diagenetic effects were minimal. We had to analyse single valves because of the extremely limited quantities of material available from the drill-core sites. Here the relevant comparison is with the $^{87}\text{Sr}/^{86}\text{Sr}$ ratio of sea water of the same date as the Miocene/Pliocene boundary, and hence the results are expressed in ΔSr units which represent deviations in parts per 10^4 from Messinian sea water²⁴.

The most complete section that has been studied is from DSDP site 376 which is in the Levantine Basin in the eastern Mediterranean. In core 376 the Sr ratios (Table 1 and Fig. 2) are relatively constant for the first 25 m just below the Pliocene/Miocene boundary (core depths of 55–80 m) with ΔSr values ranging from -3.8 to -5.2 . In the same depth interval (level 6-4/101, which is equivalent to our sample M16) the controversial 'mystery sapropel' occurs. This consists of reworked giant *Orbulina* specimens together with other planktonic foraminifers and *Cyprideis* ostracods. The nearly constant ΔSr values for samples in this depth interval (55–80 m) is consistent with a sapropel consisting of locally reworked ostracods and foraminifers from slightly older layers. At ~ 100 -m depth (~ 45 m below the Miocene/Pliocene boundary) the ΔSr values increase to values essentially identical to Messinian sea water ($\Delta\text{Sr} \sim 0$). This change in Sr isotopic compositions also coincides with a lithological transition from dolomitic marlstone with sapropelic layers to sandstone with interbedded marlstone. At that particular transition, truly freshwater ostracods (such as *Herpetocypris* and *Metacypris* juveniles, some *Candonopsis* and possibly *Ilyocypris*) were identified together with *Cyprideis*. The

presence of these freshwater species excludes the presence of marine water during the lifetime of the ostracods, whereas the Sr isotopic composition indicates host water with a marine ancestry. This dilemma can be resolved by assuming that the ionic components of the early lacustrine water were themselves derived largely from evaporated brines of marine origin and thus obtained a (Miocene) marine Sr isotopic imprint. Alternatively there may have been a transient input of marine water which dominated the Sr isotopic budget for a short period but had little overall effect on the lacustrine environment.

At the base of core 376 (samples M33 and M40) intermediate ΔSr values of -2.8 to -2.5 are found together with Sr/Ca ratios²⁵ approaching seawater values, whereas at the top of the core, near the Miocene/Pliocene boundary, Sr/Ca ratios lower than contemporaneous sea water are present. Thus, the more negative ΔSr values found in the upper section of the core indicate a trend towards low-salinity continental-derived waters (but still NaCl dominant), whereas the less negative values lower in the section (just above the uppermost gypsum layers) must have been in contact with hypersaline solutions. The presence of marls together with sapropelic layers in the upper section of core 376 (core depth ~ 50 – 80 m) also indicates an obvious hydrological transition from salt precipitation in shallow salinas to further dilution by waters with a more obvious continental isotopic imprint.

We have also analysed ostracods from the DSDP core 129A collected in the western Levantine Basin. The ΔSr values fall in the narrow range -4.6 to -5.0 which is the same as that found

TABLE 1 Sr isotope data for Mediterranean Messinian ostracods

Sample	Sample core/section-interval (cm)	Depth* (m)	Sr/Ca†	$^{87}\text{Sr}/^{86}\text{Sr}$	$\Delta\text{Sr}(t)‡$
DSDP site 376					
M16	6-4/110–111	54.5	0.00268	0.708689 ± 32	-3.8 ± 0.5
M20 I	6-4/130–131	54.8	0.00296	0.708635 ± 27	-4.6 ± 0.4
II				0.708622 ± 31	-4.8 ± 0.4
III				0.708625 ± 29	-4.7 ± 0.4
M24 I	9-1/65–66	74.6	0.00237	0.708610 ± 28	-4.9 ± 0.4
II				0.708632 ± 29	-4.6 ± 0.4
M26	9-3/40–42	77.4	0.00286	0.708621 ± 25	-4.8 ± 0.4
M30 I	9-3/136–138	78.3	0.00280	0.708592 ± 26	-5.2 ± 0.4
II				0.708612 ± 29	-4.6 ± 0.4
M31 I	9-3/138–140	78.32	0.00276	0.708677 ± 30	-4.0 ± 0.4
II				0.708675 ± 29	-4.0 ± 0.4
M33 I	11-3/100–102	100	0.00271	0.708952 ± 34	-0.1 ± 0.4
II				0.708881 ± 19	-1.1 ± 0.3
M40 I	14/CC	131	0.00390	0.708762 ± 23	-2.8 ± 0.3
II				0.708781 ± 26	-2.5 ± 0.4
DSDP site 129A					
M34 I	2/CC			0.708606 ± 27	-5.0 ± 0.4
II				0.708606 ± 11	-5.0 ± 0.2
M3B I	2/CC			0.708629 ± 17	-4.7 ± 0.2
II				0.708633 ± 21	-4.6 ± 0.3
DSDP site 372					
M9 I	4-2/90–92	152	0.00320	0.708659 ± 10	-4.2 ± 0.2
II				0.708639 ± 28	-4.5 ± 0.4
III				0.708757 ± 14	-2.9 ± 0.2
Spain					
Dk75059 I			0.00464	0.709131 ± 23	2.4 ± 0.3
II				0.709066 ± 26	1.5 ± 0.4
III				0.709084 ± 22	1.7 ± 0.3
IV				0.709108 ± 21	2.1 ± 0.3
Cyprus					
GG-Cy-6 I			0.00293	0.708823 ± 23	-1.9 ± 0.3
II				0.708950 ± 20	-0.1 ± 0.3

Sr isotope ratios were measured on individual valves using techniques described in ref. 12. Measurements were made using a MAT261 with a value for NBS 987 = 0.710200 ± 10 .

* True depth in core (from ocean floor).

† Mean Sr/Ca atomic ratios for individual horizons indicating that the ostracod valves did not undergo diagenesis. For further details see ref. 25.

‡ $\Delta\text{Sr} = [(^{87}\text{Sr}/^{86}\text{Sr})_{\text{meas}}(t)] / [(^{87}\text{Sr}/^{86}\text{Sr})_{\text{sea water}}(t)] - 1$ where $t = 5$ Myr and $(^{87}\text{Sr}/^{86}\text{Sr})_{\text{sea water}}(t) = 0.708960$ (ref. 24).

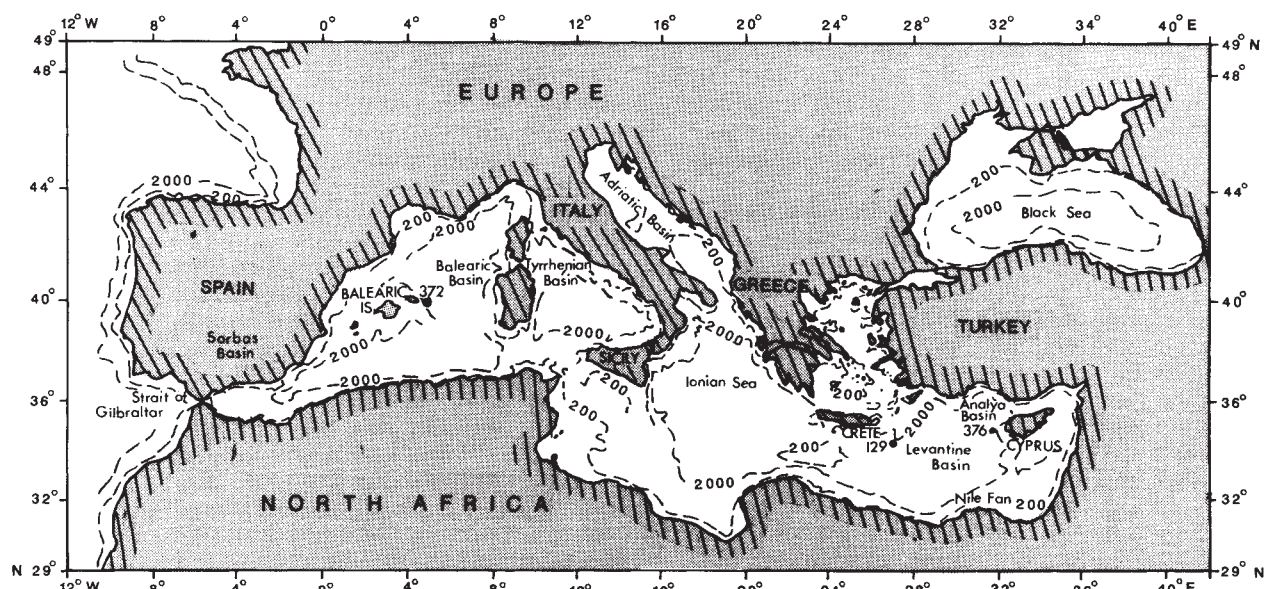


FIG. 1 Map of the Mediterranean Basin showing the location of the DSDP drill holes and the onshore sample sites. Ostracods from the Balearic Basin in the west and the Levantine Basin in the east have similar Sr isotopic

compositions indicating the presence of a large (>2,000 km) interconnected lacustrine system.

in the upper 80 m of DSDP site 376 from the same basin. In addition three valves were analysed from DSDP site 372 in the Balearic basin of the western Mediterranean (see Fig. 1). Two of the valves have negative ΔSr values falling within the same narrow range (-4.2 to -4.5) as found in the upper section of sites 376 and 129A from the Levantine Basin. The slightly discordant value of -2.9 ± 0.2 (sample M9C) is identical to that found at the base of core 376. The similar and limited range of negative ΔSr values provides strong evidence for a direct connection between some of the major Messinian, Lago Mare Basins (that is, the Balearic and Levantine Basins), despite the fact that they were separated by distances of over 2,000 km. It is therefore likely that the inland sea, the Paratethys, must have extended as far as the Mediterranean Basin.

The two onshore samples of Messinian age from Cyprus^{25,26} also have negative ΔSr values; one value is identical to that Messinian sea water. This is within the same range of values as observed at the base of core 376. In contrast to these results, ostracods from the Messinian of southeastern Spain from the 'white lagoonal limestone' (that is, the white layer which represents the terminal Messinian)^{4,5,27} of the Sorbas Basin have positive ΔSr values of 1.5 to 2.4. These values are markedly different from those of the major Lago Mare Basins and clearly imply that the Sorbas Basin had a different continental input compared to the major Mediterranean Basins, including the adjacent Balearic Basin. The distinctive ΔSr values indicate, contrary to what is observed in the other Messinian Mediterranean basins, that the Sorbas Basin was a restricted (barred) basin isolated from the rest of the Mediterranean after deposition of the gypsum evaporitic beds. The Sr/Ca ratios in ostracods from the Sorbas Basin form a broad range; some of the values are different from typical Messinian marine waters. This further confirms the independent conclusion²⁷ based on the Zorreras Member from the Sorbas Basin (within which the white lagoonal limestone occurs) which was deposited at the time of closure of the Betic Straits.

Our results for sites 376 and 129A in the Levantine Basin, site 372 from the Balearic Basin and the onshore samples from Spain and Cyprus, have a number of important implications for the palaeoenvironment of the Messinian Mediterranean. The exceedingly narrow range of negative ΔSr values provides good evidence for a direct connection between the widely separated

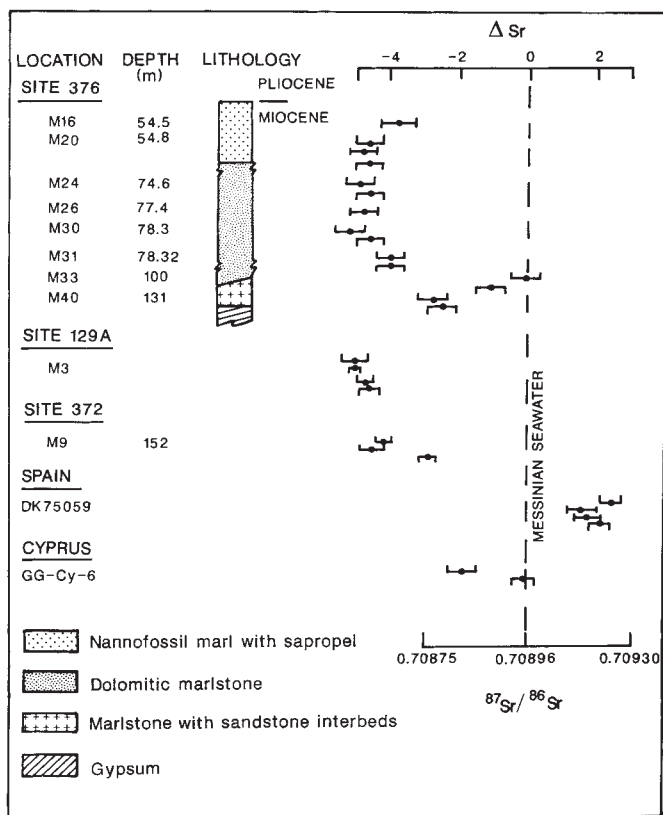


FIG. 2 Graph showing the Sr isotopic composition as a function of depth (in metres below the sea floor) for individual ostracod valves from several DSDP drill-core sites and onshore sites in the Mediterranean basin. The ΔSr values represent deviations in parts per 10^4 of the $^{87}Sr/^{86}Sr$ ratio from the value for Messinian sea water²⁴.

large basins of the Lago Mare in the Mediterranean, immediately before the Pliocene transgression. The Sorbas Basin on the western edge of the Mediterranean seems to be an exception. These observations are consistent with the deep-basin model^{1-4,28} of the Messinian Mediterranean, in which a large

'inland sea' (the Lago Mare^{6,8,11}) formed that was largely isolated from the open-marine environment at the end of the Messinian. Thus the 'wet' Mediterranean theory²⁹ may have some relevance for the Lago Mare phase, but it is clear that a lacustrine, not a marine, environment existed. A lacustrine environment close to freshwater is indicated by the Mg/Ca ratios *Cyprideis* ostracods²⁵ as well as by the presence of truly freshwater ostracod taxa.

The obvious lithological change from sandstones to marls with sapropelic layers coinciding with low Sr/Ca ratios (Table 1) and a change in ΔSr values of the ostracod shells, indicates an influx of fresh (or brackish) continental water at site 376. It has been proposed¹ that the source of this water is the inland Paratethys Sea of eastern Europe. To account for the negative ΔSr values the inflowing waters must also have ΔSr values as, or more negative, than those observed. This could be the result of dissolution of Sr from young volcanic provinces or, more probably, from older limestones. From the Sr-isotope seawater curve¹³⁻¹⁵, lower Tertiary or limestones as old as Ordovician would be suitable sources. Another possible source is the Nile River. At present the Nile has a very negative ΔSr value ($\Delta Sr \sim -60$)^{30,31} and its waters could rapidly lower the Sr isotopic composition of the Mediterranean Lago Mare lacustrine waters. The narrow range of only slightly negative ΔSr values that has been observed for the widely separated sites, 376 and 372, however, suggests that it is unlikely that Nile River water was a significant input to the Mediterranean Basin. The seawater-like ΔSr values at the bottom of core 376 and in the Cyprus sample, together with the presence of ostracods of lacustrine affinity, may be due to a transient marine influx but is more probably due to redissolved, evaporated brines containing Sr that had a marine origin. Throughout this period (latest Messinian) the ostracods indicate that the waters in the Lago Mare remained NaCl-dominant (that is, chemical pathway II³²), even in their dilute stage, as shown by the presence of *Cyprideis* ostracods which have a preference for this type of water³³. □

Hydrothermal oil of Guaymas Basin and implications for petroleum formation mechanisms

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PETROLEUM-LIKE hydrocarbons have been detected in thermally altered Recent sediments of Guaymas Basin¹⁻⁵ and petroleum-like hydrocarbon impregnations were found in hydrothermal mounds on the sea floor and associated with hydrothermal vent emissions⁵⁻⁹. Here we report the evaluation of such a hydrothermal oil, which we find to be similar to conventionally exploited crude oils. Its young geological age (<5,000 yr, ¹⁴C)¹⁰ indicates that a significant fraction of the organic carbon in the oil has completed the transformation from biomass to migrating oil in less than 5,000 years, thus limiting the oil generation, expulsion and migration processes to a geologically short timescale. We estimate the generation potential of such hydrothermal oil and discuss its implications to our understanding of the petroleum generation, expulsion and migration mechanisms.

Guaymas Basin, located in the central part of the Gulf of California, is part of a rift zone that links the East Pacific Rise in the south with the San Andreas Fault leading north. The tectonic graben-like basin is covered by thick layers of sediments (up to ~500 m), consisting of diatomaceous ooze and terrigenous silty mud^{11,12}. Hydrothermal activity in the area was postulated on the basis of anomalously high heat flow^{11,13,14} and then detected by instrument-assisted observations¹⁵. Later, diving with the submersible *Alvin* allowed direct sampling and gathering of visual evidence on the hydrothermal systems of Guaymas Basin¹⁶⁻¹⁸.

The hydrothermal activity in Guaymas Basin releases^{17,19-21} discrete oil globules (up to 1-2 cm in diameter), which are discharged into the water column with the hydrothermal fluids. The disturbance of the surface layers of bottom sediments and chimney debris also releases oil globules. Direct measurement of the oil flow rate at the vent sites has so far not been feasible, but the *in situ* collection of oil globules showed that the gas/oil ratio is ~5, with possible gas dissolution in hydrothermal water and seawater exposure of the oil before and during expulsion from the vent system²². Low bottom temperatures (2-3 °C) contribute to condensation and retention of some of the oil and this generates oil impregnations on and in inorganic substrates of the hydrothermal vent systems.

A hydrothermal chimney (40-cm length × 8-12 cm diameter; ~1,500 g), was removed from a vent that was releasing hydrothermal fluids (translucent water, at ~200 °C) at the moment of sampling (DSV *Alvin* Dive 1630, Fig. 1 insert) at 2,020-m water depth^{20,22}. At the surface the sample was sealed in double, pre-washed Kapak bags and frozen (-40 °C) until analysis.

The hydrothermal chimney consisted of a porous unconsolidated inorganic matrix (57.0%), water (19.2%) and a high organic matter content (23.8%). On thawing the sample released 2.1% of free-flowing oil. The vapour phase in contact with the sample was analysed for light hydrocarbons and alkanes from methane (15.5%) to hexadecane (0.7%) were detected. Selected hydrocarbon ratios²² are similar to those of the gasoline fraction of typical crude oils. The general distribution pattern of light volatile hydrocarbons in this chimney resembles that of crude oils, with the exception of a lower content of aromatic hydrocarbons. This is probably caused by water washing and results from the sample-water contact during formation of the chimney, sample collection and retrieval from the sea floor (~2,000-m depth). No olefins were detected among the volatile

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1. Hsü, K. J. *et al.* *Nature* **267**, 399-403 (1977).
2. Hsü, K. J. *et al.* *Init. Rep. DSDP Leg. 42*, 219-304 (1978).
3. Melières, F., Chamky, H., Coumes, F. & Rouge, P. *Init. Rep. DSDP Leg. 42*, 361-384 (1978).
4. Roep, T. B. & van Harten, D. *Annls Géol. Pays Hell. Tome hors serie III*, 1037-1044 (1979).
5. Dronkert, H. *Memorie Soc. geol. ital.* **16**, 341-361 (1976).
6. Cita, M. B., Wright, R. C., Ryan, W. B. F. & Longinelli, A. *Init. Rep. DSDP Leg. 42*, 1003-1035 (1978).
7. Drooger, C. W. (ed.) *Messinian Events in the Mediterranean* (North Holland, Amsterdam, 1973).
8. Hsü, K. J. *et al.* *Init. Rep. DSDP Leg. 42*, 1-1249 (1978).
9. McKenzie, J. A. & Ricchiuto, T. E. *Init. Rep. DSDP Leg. 42*, 650-655 (1978).
10. Pierre, C. & Fontes, J. C. *Init. Rep. DSDP Leg. 42*, 635-650 (1978).
11. Ruggieri, G. *Publs Syst. Ass.* **7**, 283-290 (1967).
12. McCulloch, M. T., De Deckker, P. & Chivas, A. R. *Geochim. cosmochim. Acta* **53**, 1703-1710 (1989).
13. DePaolo, D. J. & Ingram, B. L. *Science* **227**, 938-941 (1985).
14. Palmer, M. R. & Elderfield, H. *Nature* **314**, 526-528 (1985).
15. Koepnick, R. B. *et al.* *Chem. Geol.* **58**, 55-81 (1985).
16. Elderfield, H. *Palaeogeogr. Palaeoclimatol. Palaeoecol.* **57**, 71-90 (1986).
17. Mulder, C. J. *Messinian Events in the Mediterranean* (ed. Drooger, C. W.) 44-59 (North Holland, Amsterdam, 1973).
18. Weijermars, R. *Tectonophysics* **148**, 211-219 (1988).
19. De Deckker, P., Colin, J. P. & Peyrouquet, J. P. (eds) *Ostracoda in the Earth Sciences* (Elsevier, Amsterdam, 1988).
20. De Deckker, P. *Palaeogeogr. Palaeoclimatol. Palaeoecol.* **62**, 463-475 (1988).
21. Turpen, J. B. & Angell, R. W. *Biol. Bull.* **140**, 331-338 (1971).
22. Chivas, A. R., De Deckker, P. & Shelley, J. M. G. *Hydrobiologia* **143**, 135-142 (1986).
23. De Deckker, P. *Hydrobiologia* **81**, 131-144 (1981).
24. McKenzie, J. A., Hodell, D. A., Mueller, P. A. & Mueller, D. W. *Geology* **16**, 1022-1025 (1988).
25. De Deckker, P., Chivas, A. R. & Shelley, J. M. G. *Palaiois* **3**, 352-358 (1988).
26. Rouchy, J. M. *Bull. Centre Rech. Expl. Elf-Aquit.* **4**, 511-545 (1980).
27. Müller, D. W. & Hsü, K. J. *Paleoceanography* **2**, 679-696 (1987).
28. Hsü, K. J. *Scient. Am.* May 53-63 (1978).
29. Dietz, R. S. & Woodhouse, M. *Geotimes* **43**, 4 (1988).
30. Brass, G. W. *Geochim. cosmochim. Acta* **40**, 721-730 (1976).
31. Palmer, M. R. & Edmond, J. M. *Earth planet. Sci. Lett.* **92**, 11-26 (1989).
32. Eugster, H. P. & Hardie, L. A. in *Saline Lakes* (ed. Lerman, A.) 237-294 (Springer, Berlin, 1978).
33. Anadon, P., De Deckker, P. & Julia, R. *Hydrobiologia* **143**, 199-208 (1986).

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hydrocarbons indicating that the organic matter had not been exposed to severe thermal cracking processes. The inorganic matrix of the chimney consisted predominantly (85%) of barium sulfate crystals²³, with the organic matter adsorbed and impregnating the interstitial pore space of the unconsolidated barite matrix.

The hydrothermal oil from the Guaymas Basin is similar to reservoir crude oils. First, the total extractable organic matter distilled over the normal range, but the gasoline fraction is low indicating that a significant portion of the volatile (C_1 – C_{10})

components were lost in the thermocline before recovery and preservation. Second, the elemental composition (Table 1a) is within the normal ranges of typical crude oils²⁴, with slightly higher contents of vanadium and nickel as we expect for a light oil with a relatively high content of intermediate distillation products. Third, the porphyrin and biomarker contents and their distribution in this sample are well within the range of normal crude oils²⁵, indicating moderate degrees of maturation and thermal stress. Fourth, the compound-class composition (Table 1a) is just like a typical reservoir crude.

TABLE 1 Hydrothermal petroleum from Guaymas Basin

(a) Characteristics					
Characteristics of organic matter		Distillation Yield			
Specific gravity (60 °F/60 °F)	0.8891	Gasoline (IBP–300 °F)	3%		
API gravity (60 °F)	27.6	Kerosene–Diesel (300–600 °F)	61%		
Conradson carbon	4.2%	Gas oil (660–1,000 °F)	21%		
Carbon	85.0%	Residue (>1,000 °F)	15%		
Hydrogen	11.1%	Composition*	Free oil	Total extract	
Nitrogen	0.49%		Paraffinic & naphthenic HC	54.5%	44.6%
Oxygen	1.50%		Aromatic HC	26.6%	34.6%
Sulphur	0.99%		N, S & O compounds	9.8%	8.8%
Phosphorus	0.005%		Asphaltenes	9.1%	12.8%
Vanadium	214 p.p.m.				
Nickel	98 p.p.m.				
Ratios (atomic)					
Carbon/Hydrogen (C/H)	0.64 (H/C=1.57)				
Carbon/Nitrogen (C/N)	142				
Carbon/Oxygen (C/O)	75				
Carbon/Sulphur (C/S)	228				
Carbon/Phosphorus (C/P)	44,250				
Vanadium/Nickel (V/Ni)	2.56				
(b) Biomarkers					
		Comments		Relative molecular mass distribution	
I	Metallo porphyrins type	Structural distribution	Carbon number		
	Vanadyl 363 p.p.m.	ETIO† > 95%	C ₂₄ –C ₃₀	366–450	
	Nickel 145 p.p.m.	DPEP‡ < 5%§	max C ₂₇	max 408	
II	n-Alkanes				
	Range	Carbon preference index	Monomodal	170–352	
	n–C ₁₂ –n–C ₂₅				
	max n–C ₁₅	C _{11–22} = 0.99			
III	n-Alkylcyclohexanes				
	C ₁₃ –C ₂₂		Monomodal	182–308	
	max C ₁₇				
IV	n-Alkylbenzenes				
	C ₁₃ –C ₂₂		Monomodal	176–302	
	max C ₁₇				
V	Isoprenoids				
	C ₉ –C ₂₀	Pristane/Phytane		128–282	
	max C ₁₉	1.85			
VI	Terpanes				
	C ₁₉ –C ₃₅	Tricyclic C ₁₉ –C ₂₉		260–482	
	max C ₃₀	Pentacyclic C ₂₇ –C ₃₅			
VII	Steranes/Diasteranes¶				
	C ₂₁ –C ₂₉			288–400	
	max C ₂₇				
VII	Unresolved complex mixture				
	n–C ₁₂ –C ₂₅ (GC retained)		Monomodal	158–348	
	max n–C ₁₇ (GC retained)				

* >200 °C fraction of oil.

† ETIO, etioporphyrins.

‡ DPEP, deoxophylloerythroetioporphyrins.

§ Conversion of DPEP to ETIO porphyrins is associated with the thermal evolution of geolipids³¹ and is also associated with a decrease in porphyrin concentration and displacement of the porphyrin-carbon-number maximum towards lower values³².

|| Other biomarkers in the hydrocarbon fraction consist of triterpenoids, extended tricyclic terpanes and steranes with their rearranged analogues (diasteranes)^{6,8,9,33}. Minor extended tricyclic terpanes range from C_{20} to C_{29} with a similar distribution pattern as observed for other mature petroleum samples. The triterpenes are essentially 'mature' in their more stable configurations and are comprised primarily of the 17α (H), 21β (H)-hopane series ranging from C_{27} (no C_{28}) to C_{35} , with the homologues from C_{31} – C_{35} present as 22-*S* and *R* epimer pairs in a ratio of about 1.3 (*S*/*R*)^{6,8}. Minor amounts of 17β (H), 21α (H)-hopanes (moretan- C_{29} , C_{30} and C_{31}) are also present.

¶ The steroidal markers primarily consist of 5α (H), 14α (H), -17α (H)-steranes (20*R*), and diasteranes mainly as the 13β (H), 17α (H)-20 *R* or *S* series. The steranes range from C_{26} to C_{30} , with cholestane as the major homologue. The distribution pattern indicates a marine autochthonous origin, an intermediate maturity and is comparable with data for other samples from the area^{6,8}.

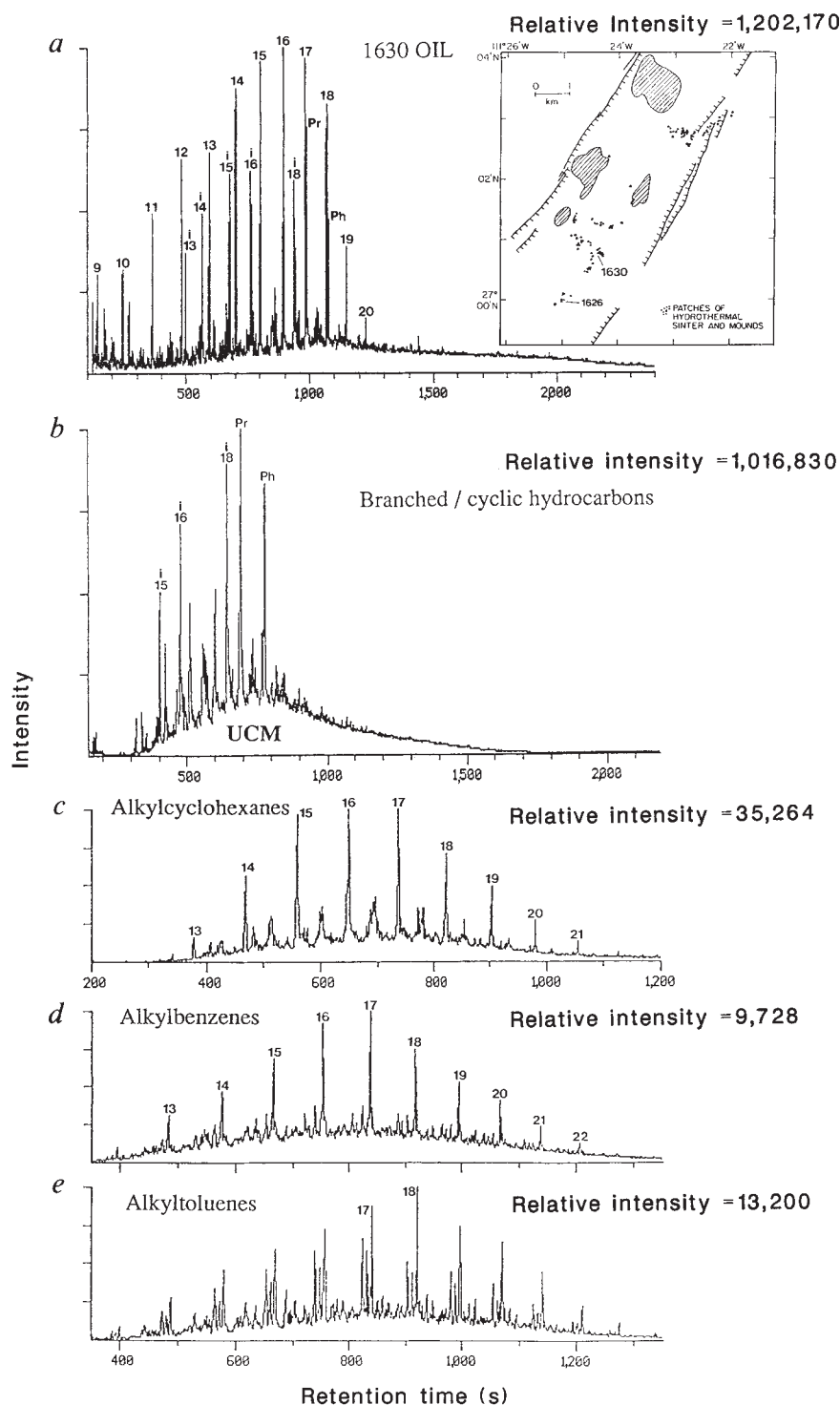


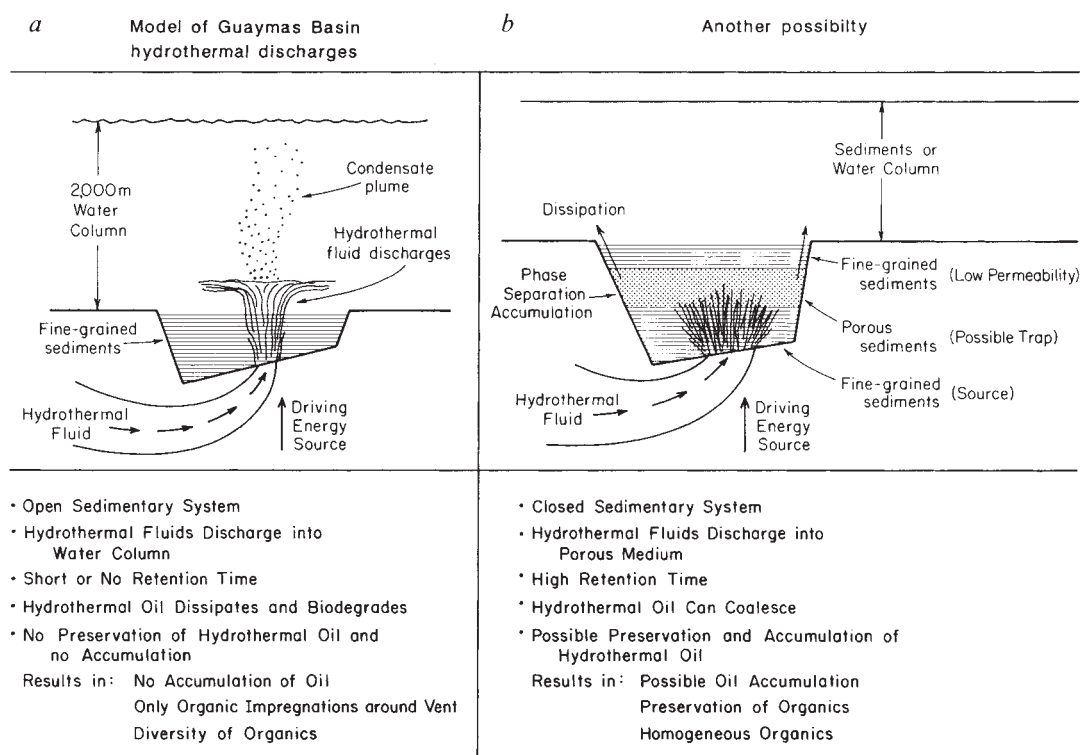
FIG. 1 Salient features of the gas chromatography/mass spectrometry data for the *a*, total and the *b*–*e*, branched–cyclic hydrocarbons of oil sample 1630 (inset in *a* location map of the dive area in the south rift of Guaymas Basin). *a*, Total ion-current trace (numbers refer to *n*-alkanes, *i* *n* to isoprenoid hydrocarbons, Pr to pristane, Ph to phytane). The *n*-alkane range for the stabilized oil (C_{12} – C_{24} , max C_{15}) has a monomodal, narrow carbon-number distribution with no carbon preference (carbon preference index C_{12} – C_{22} = 0.99), compatible with a predominantly bacterial/algal origin for the source organic matter and with an adequate degree of maturation. *b*, Total ion-current trace (*i* *n*, isoprenoid hydrocarbons; UCM, unresolved complex mixture, that is, naphthenes). A range of isoprenoid hydrocarbon biomarkers is present, C_{15} – C_{20} (no C_{17}) and pristane as the dominant homologue. *c*, m/z = 83, key ion for alkylcyclohexanes (numbers indicate carbon number of homologues). *d*, m/z = 91, key ion for alkylbenzenes. *e*, m/z = 105, key ion for alkyltoluenes. On the whole, these distributions are compatible with an algal/bacterial origin for the organic matter.

The gas chromatography/mass spectrometry data (Fig. 1) are compatible with a predominantly bacterial/algal origin for the source organic matter and suggest an adequate degree of maturation.

Sediments have been accumulating in the rift areas of Guaymas Basin for ~150,000 yr at an assumed rate of 0.2 cm yr^{-1} and this, combined with turbidite flows, has resulted in a ~400-m layer of diatomaceous ooze¹¹. Thus, if the oil associated with these vent systems has not originated outside the rift areas and is, as indicated by previous geological and geochemical evidence^{11,17}, autochthonous from the Guaymas Basin sediments, it has probably formed by the action of hydrothermal

processes on the organic matter of these relatively Recent sediments^{4,5,7}. Radioactive-carbon isotope (^{14}C) dating of the oil from this and a nearby area (dives 1630 and 1626) gives ages of $4.2\text{--}4.9 \times 10^3$ yr with uncertainties in the range 50–190 yr (ref. 10). Therefore, in Guaymas Basin, the overall time–temperature conversion of sedimentary organic matter to hydrothermal petroleum takes place over a short geological timescale (<5,000 yr) and commences under relatively mild temperature conditions, as indicated by the presence and characteristics of the biomarkers. The oil expulsion and migration mechanisms are provided by the hydrothermal fluids under pressures of 200 bars and temperature conditions up to and exceeding 315 °C

FIG. 2 Schematic models for hydrothermal petroleum generation and migration scenarios: *a*, Guaymas Basin open system (in excess of ~200 bar). *b*, Hypothetical closed system.



at some vent outlets. There are probably near-critical conditions further down in the sedimentary column^{8,26,27}.

The hydrothermal petroleum is discharged into the water column, where it is exposed to water washing, dispersion and active biodegradation. This is supported by the compositional data for petroleum impregnations with different degrees of environmental exposure and biodegradation in the Guaymas Basin area⁶⁻⁸. Thus, hydrothermal-oil transport and release results in its dispersion, alteration and eventual loss. A schematic representation of the prevailing conditions in the Guaymas Basin

TABLE 2 Hydrothermal petroleum potential of Guaymas Basin (South Trough)

Basic considerations used for estimate:

Area of hydrothermal activity presently known	~3 × 10 km = 30 km ²
Sedimentary layer	~300–500 m* = 400 m
Hydrothermally altered sedimentary layer	lower 120 m* = 120 m
Porosity	40–80%* = 50%
Organic carbon (dry weight, unaltered section)	av. 2%† = 2%
Organic carbon (altered section)	decreases to 0.2%† = 0.2%
Estimates:	
Sediment volume (dry basis)	= 2 × 10 ⁸ m ³ km ⁻²
Sediment weight (dry, used density 1 g cm ⁻³)	= 2 × 10 ⁸ t km ⁻²
Organic carbon converted (2% to 0.2% = 1.8%)	= 1.08 × 10 ⁶ t OC km ⁻²
Organic matter (oil = 85% TOC)	= 1.3 × 10 ⁶ t oil km ⁻²
Total oil in altered section of area (30 km ²)	= 40 × 10 ⁶ t

* Compare to data in ref. 11.

† Compare to data in ref. 32.

systems²⁸ is shown in Fig. 2a. Hydrothermal fluids driven by a deep heat source, permeate through an open, fine-grained body of Recent sediments and discharge directly into the water column. The oil discharged with the hydrothermal fluids partially adsorbs or condenses on inorganic substrates cooled by sea water (~3 °C) surrounding the vents. The major part of the oil plume above the vent area dissipates into the water column mainly by dispersion, dissolution and eventual biodegradation. Another scenario could be postulated (for example, Fig. 2b) where a similar hydrothermally generated oil is discharged into a porous sedimentary body, with a finite retention time for the fluids. There the hydrothermal oil-water mixtures can undergo phase separation and petroleum could eventually accumulate if adequate sedimentary and tectonic features are available.

In Guaymas Basin the presently known area where hydrothermal activity reaches the sea floor is ~3 × 10 km and the lower 120-m section of the sediments drilled was hydrothermally altered¹¹. An average porosity of ~50% and an organic carbon decrease from 2.0 to 0.2% (typical of the area²⁹) give a value for total organic matter (oil potential) susceptible to hydrothermal alteration and fluid leaching of the order of 1.3 × 10⁶ t km⁻² of alteration zone (Table 2). This gives a maximum organic-matter availability of ~40 × 10⁶ t for the presently known hydrothermally active area of the basin (Table 2). So far no data is available on the ultimate efficiency of hydrothermal petroleum generation processes prevailing in Guaymas Basin. The combination of field data with a conservative potential-yield calculation, however, indicate a high efficiency and significant quantities of hydrothermal oil could have been generated by this relatively small area during its geologically active lifetime. If larger areas and volumes of sediments were involved (where there is, at present, no evidence that hydrothermal activity reaches the sea bottom or if larger hydrothermally altered areas have formed because of hot-spot displacement along the tectonic axis of the graben during its geologic evolution) the overall hydrothermal-petroleum generating potential of the area could be considerably larger.

The generally accepted model of oil generation, postulated to be responsible for the formation of the major part of the presently exploited petroleum, assumes long-term maturation

of sedimentary organic matter in subsiding sedimentary basins, where the organic matter undergoes successive and gradual increases in geothermal alteration leading to a process of continuous oil generation. The expulsion of the oil and its subsequent migration occur later and only after the geothermal maturation of the original organic matter. This multistep oil formation process has a low efficiency and converts only a minor fraction of the original organic matter of the sediment to oil. There is also difficulty in balancing and timing an adequate degree of geothermal maturation (oil generation) occurring at intermediate stages of basin evolution and ample fluid availability (expulsion), with the retention of sufficient intergrain porosity for adequate fluid transport (migration).

Although considerable progress in the understanding of this multistep oil formation mechanism has been achieved, there are still problems that need to be solved³⁰. Such a slow multistep mechanism differs significantly from hydrothermal petroleum formation, which can be geologically fast (here 5,000 yr) indicating a single-step mechanism in which generation occurs almost concurrently with expulsion and migration. In addition the whole hydrothermal process can take place during the early stages in the geological history of a basin.

Although hydrothermal oil formation has been detected, identified and documented, no evidence is so far available on the extent to which this alternative single-step oil generation process has contributed towards the origin of presently exploited oil reserves. As no hydrothermal-oil reservoirs have yet been identified, a trapping mechanism is not known, but could proceed according to the scenario described in Fig. 2b. Nevertheless, hydrothermal oil generation occurs and could result in oil accumulations under favourable geological constraints. Hydrothermal oil formation provides an efficient single-step mechanism for petroleum generation, expulsion and migration which could have a considerable impact on our understanding of petroleum formation mechanisms and eventually assist us in tapping resources in new areas. □

Evidence for anoxygenic photosynthesis from the distribution of bacteriochlorophylls in the Black Sea

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THE contribution of anoxygenic photosynthesis to carbon cycling in the Black Sea, the world's largest body of anoxic marine water, has been vigorously investigated and debated for over four decades¹⁻⁶. Penetration of light into the sulphide-containing deep water may result in a zone of anaerobic primary production by photosynthetic bacteria. We report here the results of analyses of photosynthetic pigments in samples of suspended particulate matter collected from two stations in the western basin of the Black Sea. Our data demonstrate high concentrations of a bacteriochlorophyll at the chemocline, and thus the potential for anoxygenic photosynthesis as a component of primary production in the carbon cycle of the Black Sea⁶. More than 95% of the pigments in the bacteriochlorophyll-maximum are accounted for by a series of aromatic carotenoids and bacteriochlorophylls-*e*, including a previously unreported geranyl ester of 4-*i*-butyl bacteriochlorophyll-*e*. The distribution of pigments is characteristic of the obligate phototrophs *Chlorobium phaeobacteroides* and *C. phaeovibriodes*. Total depth-integrated bacteriochlorophyll at one station exceeded total chlorophyll-*a* in the overlying oxygenated portion of the euphotic zone. We suggest that anoxygenic photosynthesis is a relatively recent phenomenon in the Black Sea initiated by shallowing of the chemocline over the past decade and development of an anoxic layer devoid of O₂ and H₂S (ref. 7).

Suspended particulate matter was collected on RV *Knorr* cruise 134, leg 9 (Istanbul to Istanbul, Turkey) in May 1988. Station BS2-1 (42°10'N, 30°00'E) is located 110 km from the Turkish coast in 2,110 m of water, and is most heavily influenced by terrigenous material advected from the shelf-slope region of the adjoining coast. Dissolved oxygen was maximal (320 µM, 5% supersaturation) at 20 m and rapidly decreased to <2 µM (limit of detection) at depths >80 m. Dissolved sulphide was detected in all the samples that were collected below 100 m. Station BS2-2 (42°50'N, 32°00'E) is located in the centre of the western basin and is characterized by a shallower chemocline. Dissolved oxygen decreased from a maximum of 307 µM at 20 m to <2 µM at >56 m. Sulphide was detected at depths as shallow as 80 m (Fig. 1). The distributions of photosynthetic pigments in the oxic portion of the water columns at each station were similar (Table 1). The major pigments included: chlorophyll-*a*, chlorophyll-*c*₁ and chlorophyll-*c*₂, which are widely distributed in marine algae; chlorophyll-*c*₃, 19'-butanoylfucoxanthin and 19'-hexanoylfucoxanthin, which are characteristic of prymnesiophytes^{8,9}; and fucoxanthin, which is widely distributed but characteristic of algae from the class Bacillariophyceae¹⁰ (Fig. 2).

Particulate matter samples collected from 90–121 m (station BS2-1) and 60–110 m (station BS2-2) all contained abundant bacteriochlorophylls and bacteriocarotenoids. Four components were isolated that had visible absorption maxima in acetone of 463 and 653 nm. The main component (45% of the total) and two lesser components (16 and 15% of the total) were identified, respectively, as the 4-ethyl, 4-*n*-propyl and 4-*i*-butyl (Fisher nomenclature) homologues of bacteriochlorophyll-*e* by mass spectrometry and comparison of chromatographic retention

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- Galimov, E. M., Kodina, L. A., Bogacheva, M. P. & Shirinsky, V. G. *Init. Rep. DSDP 64(II)* 819–836 (1982).
- Simoneit, B. R. T. & Philp, R. P. *Init. Rep. DSDP 64(II)* 883–904 (1982).
- Simoneit, B. R. T., Mazurek, M. A., Brenner, S., Crisp, P. T. & Kaplan, I. R. *Deep-Sea Res.* **A26**, 879–891 (1979).
- Simoneit, B. R. T. in *The Role of Heat in the Development of Energy and Mineral Resources in the Northern Basin and Range Province*, Geotherm. Res. Coun., Spec. Rep. 13, 215–241 (Davis, California, 1983).
- Simoneit, B. R. T., Philp, R. P., Jenden, P. D. & Galimov, E. M. *Org. Geochem.* **7**, 173–205 (1984).
- Kawka, O. E. & Simoneit, B. R. T. *Org. Geochem.* **11**, 311–328 (1987).
- Simoneit, B. R. T. in *Hydrothermal Processes at Seafloor Spreading Centers* (eds Rona, P. A. et al.) 453–474 (Plenum, New York, 1984).
- Simoneit, B. R. T. *Can. J. Earth Sci.* **22**, 1919–1929 (1985).
- Simoneit, B. R. T. & Lonsdale, P. F. *Nature* **295**, 198–202 (1982).
- Peter, J. M., Kawka, O. E., Scott, S. D. & Simoneit, B. R. T. *Third Chem. Congr. North America*, Toronto abstr. GEOC 036 (1988).
- Curry, J. R. et al. *Init. Rep. DSDP 64(II)* (1982).
- Koski, R. A., Lonsdale, P. F., Shanks, W. C., Berndt, M. E. & Howe, S. S. *J. geophys. Res.* **90**, 6695–6707 (1985).
- Lawver, L. A. & Williams, D. L. *J. geophys. Res.* **84**, 3465–3478 (1979).
- Williams, D. L., Becker, K., Lawver, L. A. & von Herzen, R. P. *J. geophys. Res.* **84**, 6757–6796 (1979).
- Lonsdale, P. F. *Eos* **61**, 995 (1980).
- Lonsdale, P. & Lawver, L. A. *Geol. Soc. Am. Bull.* **91**, 555–591 (1980).
- Lonsdale, P. *Am. Ass. Petrol. Geol. Bull.* **69**, 1160–1180 (1985).
- Lonsdale, P. & Becker, K. *Earth planet. Sci. Lett.* **73**, 211–225 (1985).
- Merewether, R., Olsson, M. S. & Lonsdale, P. *J. geophys. Res.* **90**, 3075–3085 (1985).
- Gieskes, J. M. et al. *Can. Mineralogist* **26**, 589–602 (1988).
- Simoneit, B. R. T. *Can. Mineralogist* **26**, 827–840 (1988).
- Simoneit, B. R. T., Kawka, O. E. & Braut, M. *Chem. Geol.* **71**, 169–182 (1988).
- Didyk, B. M. & Simoneit, B. R. T. *Third Chem. Congr. North America*, Toronto abstr. GEOC 035 (1988).
- Tissot, B. P. & Welte, D. H. *Petroleum Formation and Occurrence: A New Approach to Oil and Gas Exploration* 2nd edn (Springer, 1984).
- Baker, E. W. & Louda, J. W. *Org. Geochem.* **10**, 905–914 (1986).
- Bischoff, J. L. & Rosenbauer, R. *J. Earth planet. Sci. Lett.* **68**, 172–180 (1984).
- Chen, C.-T. A. *Science* **211**, 298 (1981).
- Scott, S. D. in *MTS-IEEE Conf. Proc. Oceans 83*, vol. 2 818–824 (IEEE, San Francisco, 1983).
- Simoneit, B. R. T. & Bode, G. R. in *Init. Rep. DSDP 64(II)* 1303–1306 (1982).
- Durand, B. *Org. Geochem.* **13**, 445–459 (1988).
- Didyk, B. M., Alturki, Y. I. A., Pillinger, C. T. & Eglinton, G. *Nature* **256**, 563–565 (1975).
- Didyk, B. M. thesis, Univ. Bristol (1975).
- Didyk, B. M. & Simoneit, B. R. T. *App. Geochem.* (in the press).

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TABLE 1 Concentration of main carotenoids and chlorophylls

Depth (m)	Fxn	19'-HFxn	Chl- <i>a</i>	GBchl	EBchl	PBchl	BBchl	IR	β -IR
<i>Station 1, 27 May 1988</i>									
10	72	121	335						
20	94	89	342						
30	99	36	322						
40	71	18	255						
50	8		58						
60	37		88						
70	73		20						
90	18		12	23	39	15	15	17	9
100	6		15	53	89	35	37	20	8
106	10		15	11	26	15	13	7	5
112	6		15	9	16	10	11	4	3
121	19		17	7	17	10	9	2	1
140	3		12					2	1
<i>Station 2, 17 May 1988</i>									
2	34	132	205						
20	50	136	273						
29	61	56	224						
38	115	30	330						
47	16		50						
56	56		21						
62	38		43						
68	40		47	153	286	98	87	25	17
74	22		34	219	428	154	139	55	22
83	26		32	170	440	148	140	93	22
92	11		15	57	113	39	35	11	7
98	20		30	61	102	34	31	22	16
110	7		10					1	0.5

Concentrations are those in suspended particulate matter samples from two stations in the Black Sea. Compound designations are as follows: Fxn, fucoxanthin; 19'-HFxn, 19'-hexanoylfucoxanthin; Chl-*a*, chlorophyll-*a*; GBchl, bacteriochlorophyll-*e* geranyl ester; EBchl, 4-ethyl bacteriochlorophyll-*e*; PBchl, 4-*n*-propyl-bacteriochlorophyll-*e*; BBchl, 4-*i*-butyl-bacteriochlorophyll-*e*; IR, isorenieratene; β -IR, β -isorenieratene. Blank spaces denote nondetectable ($<1 \text{ ng l}^{-1}$) pigments.

times (HPLC) with authentic standards. The fourth component (23% of the total) did not elute with any known bacteriochlorophyll-*e*. The chromatographic and spectroscopic properties of the compound initially indicated that the compound was a diastereomer or the 4-ethyl, 5-methyl homologue of bacteriochlorophyll-*e*. Diastereomers of bacteriochlorophyll-*e* have been isolated from two strains of (cultured) *Chlorobium phaeovibrioides*¹¹, but separation of these diastereomers by using a

reverse-phase HPLC system similar to the one used here was not achieved, and it seemed unlikely that the modifications to our chromatographic analyses would have achieved such a separation. The chemical ionization (methane) mass spectrum of the pure bacteriophageopigment that was derived from the unidentified component displayed a molecular ion at $m/z = 758$ ($M+1$) with major fragment ions at $m/z = 740$ ($M+1$ - water), 622 and 604 (Fig. 3). Major fragment ions at $m/z = 622$ (macrocycle) and 604 (macrocycle-water) were also present in the mass spectrum of 4-*i*-butyl-bacteriophageofarnesyl-*e* (derived from 4-*i*-butyl-bacteriochlorophyll-*e*). The two compounds share a common macrocycle, but differ in the esterifying alcohol. Other bacteria of chlorobiaceae are known to produce bacteriochlorophyll-*c* and -*d* with a suite of esterifying alcohols. Only the farnesyl ester of bacteriochlorophyll-*e*, however, has been reported. The mass difference of 68 AMU between the molecular ions of 4-*i*-butyl-bacteriochlorophyll-*e* and the new bacteriochlorophyll in our sample corresponds to a difference of C_5H_8 (isoprene) in the esterifying alcohol. Therefore, we tentatively identify the new bacteriochlorophyll-*e* in our samples as the geranyl ester of 4-*i*-butyl-bacteriochlorophyll-*e*. It is interesting to note that, although the ethyl, *n*-propyl, and *i*-butyl homologues of bacteriochlorophyll-*e* occurred in nearly constant proportions in all our samples (irrespective of depth or station location), only the *i*-butyl homologue of geranyl-bacteriochlorophyll-*e* was detected.

Concentrations of total bacteriochlorophyll-*e* reach a maximum of 214 ng l^{-1} at 100 m (station BS2-1) and 940 ng l^{-1} at 74 m (station BS2-2) (Table 1). The lower concentration of bacteriochlorophyll-*e* at station BS2-1 is associated with a deeper chemocline and a lower ambient light intensity. From the relative distribution of pigments at the two stations, bacterial growth seems to be limited by penetration of light into the sulphide-containing deep water. A 0.1%-light-level depth of

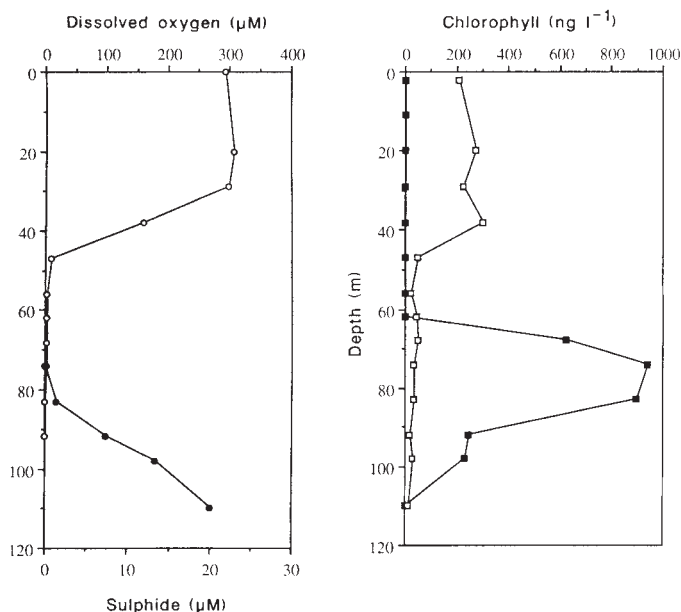


FIG. 1 The distribution of chlorophyll-*a* (□), total bacteriochlorophyll-*e* (■), dissolved oxygen (○), and hydrogen sulphide (●) at station 2 on 17 May 1988.

30–40 m was estimated from *in situ* productivity measurements¹⁴. Total depth-integrated bacteriochlorophyll-*e* at station BS2-2 exceeds total chlorophyll-*a* from phytoplankton by a factor of two (Table 1). It is difficult to translate our chlorophyll values into biomass (carbon), because particulate organic carbon (POC) at the bacteriochlorophyll maximum (station BS2-2) could contain significant contributions from abundant heterotrophic bacteria^{14,15}. The ratio of POC to bacteriochlorophyll averaged 22 ± 2 at 83 m, compared with a POC: chlorophyll-*a* ratio of 25 at the base of the oxic euphotic zone (at 55 m). Assuming a POC: bacteriochlorophyll-*e* ratio of 22 for the entire 30-m depth of the secondary chlorophyll maxima, and integrating for total bacteriochlorophyll from the data presented in Table 1, we estimate a total photosynthetic bacterial biomass of 0.5 g m^{-2} , compared with a total phytoplankton biomass of 0.9 g m^{-2} for 0–60 m. Photosynthetic bacteria therefore comprise a significant fraction of the photosynthetically active biomass in the euphotic zone, and have the potential to supply a significant fraction of the total primary productivity.

The pigment complement of our chemocline samples are characteristic of the brown sulphur bacteria *Chlorobium phaeovibrioides* and *C. phaeobacteroides*^{16–18}. In culture these bacteria are non-motile, 1–3- μm long and require reduced sulphur compounds (H_2S , S^0) for growth. Their *in vivo* absorption is between 450 and 460 nm and between 700 and 760 nm, and the organisms are capable of growth at very low light intensities¹⁷. Seasonal blooms of *C. phaeobacteroides* have been frequently reported in shallow, stagnant freshwater lakes where sulphide-containing bottom water penetrates the euphotic zone^{19,20}. The minimum

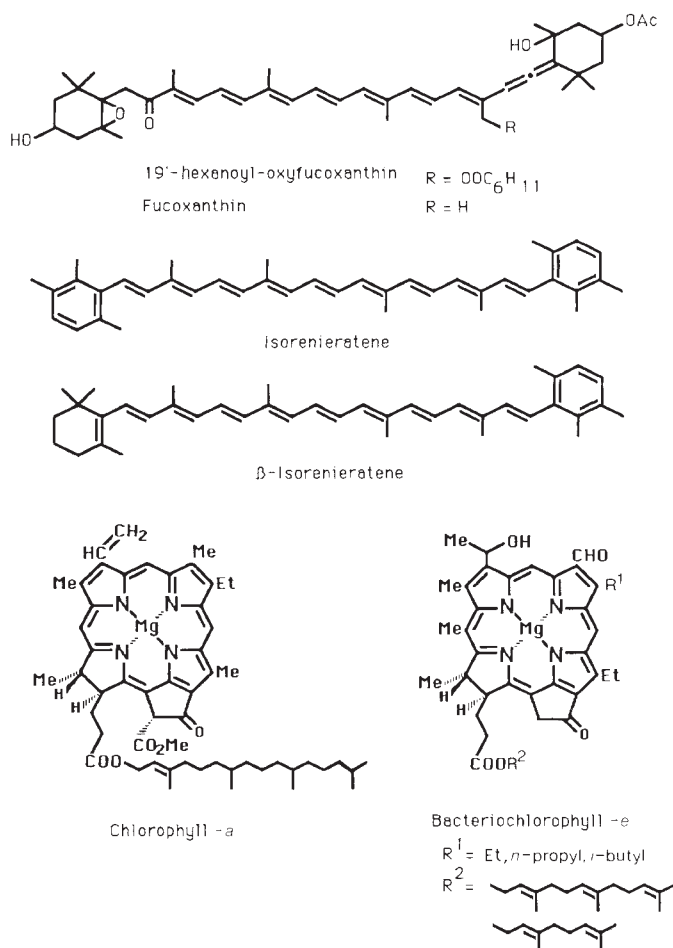


FIG. 2 Structures of pigments discussed in the text; concentrations are given in Table 1.

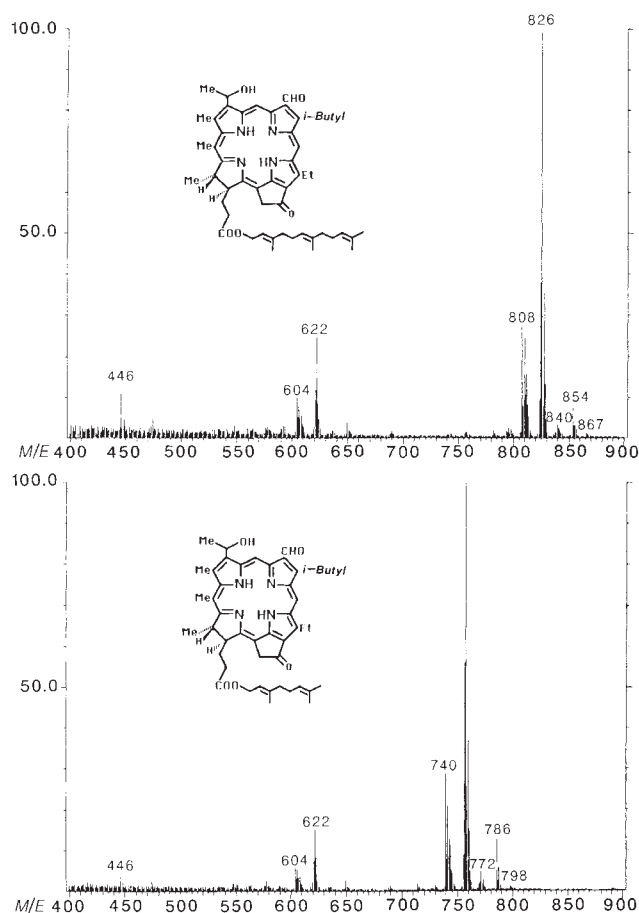


FIG. 3 Chemical ionization mass spectra of 4-*i*-butyl-bacteriopheofarnesyl-*e* (top) and 4-*i*-butyl-bacteriopheoeranyl-*e* (bottom) isolated from suspended particulate matter at station 2 (83 m). Vertical axis is intensity on a scale 0–100. Pigments (obtained by treatment of bacteriochlorophylls with acid) were purified by HPLC, evaporated onto a gold-tipped direct insertion probe and introduced to the mass spectrometer. Spectra were collected by heating the probe ballistically to 300 °C over 10–15 s to desorb the pigments.

depth of *Chlorobium* growth in the Black Sea is restricted by the availability of reduced sulphur compounds that are needed to sustain photosynthetic activity. At both stations, the depth of the bacteriochlorophyll maximum corresponds to the first appearance of sulphide (Fig. 1). The rate of H_2S oxidation was greatest at the base of the bacteriochlorophyll maximum (station BS2-2), but we observed no significant light-induced rate enhancement in one *in situ* incubation on May 26. Dissolved sulphide gradients measured simultaneously with our incubation of May 26 show that the interface was strongly disturbed at the time of sampling, and that additional measurements are necessary to quantify bacterial photosynthetic rates *in situ*. From our preliminary experiments, measured rates of sulphide oxidation do not seem to follow the distribution of photosynthetic bacteria, indicating very low rates of photosynthetic activity. More studies, however, are needed to establish the rate of anaerobic photosynthesis and assess its relative contribution to total primary productivity.

The role of the anaerobic photosynthesis in the Black Sea has been the subject of debate for nearly four decades. Previous reports of high concentrations of viable phototrophic sulphur bacteria at depths >160 m implied that anaerobic photosynthesis could be an important route for carbon fixation^{1–3}. The mass mortality of photosynthetic bacteria from seasonal deepening of the chemocline was further cited as a possible source for deep sea varves³. Yet in the spring of 1975, Jannasch *et al.*⁴ and

Hashwa and Trüper⁵ did not find viable photosynthetic bacteria in Black Sea water sampled in and below the chemocline, which was then at a depth of 140 m. Isolates of photosynthetic bacteria from sediment (collected at 2,000 m) were attributed to survival as chemo-organotrophs on dissolved organic matter. Our results signify that at the H₂S interface depth of 80–100 m that exists now, anaerobic photosynthesis has become a significant process in the Black Sea carbon cycle. The shallowing of the chemocline was accompanied by the separation of the oxygenated upper

layer and the shallowest appearance of H₂S by a suboxic layer (<5 µM O₂) of 20–30 m (Fig. 1). Considerations of possible electron acceptors for anaerobic H₂S oxidation support the phototrophic reduction of CO₂ to organic carbon. Thus, the Black Sea supports the largest and deepest extant community of anoxygenic photosynthetic bacteria in the world, and may serve as a suitable model for studies of carbon recycling in the suboxic and anoxic seas of Cambrian and Precambrian times. □

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- Kriss, A. E. & Rukina, E. A. *Dokl. Akad. Nauk, SSSR* **93**, 1107–1110 (1953).
- Sorokin, Y. I. *J. Cons. Int. Explor. Mer.* **29**, 41–53 (1964).
- Dickman, M. & Artuz, I. *Nature* **275**, 191–195 (1978).
- Jannasch, H. W., Trüper, H. G., and Tuttle, J. H. in *The Black Sea—Geology, Chemistry and Biology* (eds Degens, E. & Ross, D.) 419–425 (Mem. No. 20, Am. Assn. Petrol. Geol., Tulsa, Oklahoma, 1974).
- Hashwa, F. A. & Trüper, H. G. *Helgoländer wiss. Meeresunters.* **31**, 249–253 (1978).
- Deuser, W. G. *Deep Sea Res.* **18**, 995–1004 (1971).
- Murray, J. W. *et al. Nature* **338**, 411–413 (1989).
- Liaaen-Jensen, S. *Pure appl. Chem.* **57**, 649–658 (1985).
- Wright, S. and Jeffrey, S. W. *Mar. Ecol. Prog. Ser.* **38**, 259–266 (1987).
- Liaaen-Jensen, S. in *Marine Natural Products* Vol. 2 (ed. Scheuer, P. J.) 2–73 (Academic, New York, 1978).

- Simpson, D. J. & Smith, K. M. *J. Am. chem. Soc.* **110**, 1753–1758 (1988).
- Caple, M. B., Chow, H. & Strouse, C. E. *J. Biol. Chem.* **253**, 6730–6737 (1978).
- Smith, K. M., Craig, G. W., Kehres, L. A. & Pfennig, N. *J. Chrom.* **281**, 209–223 (1983).
- Karl, D. M. & Knauer, G. A. *EOS* **69**, 1241 (1988).
- Wirsén, C. O. and Jannasch, H. W. *EOS* **69**, 1241 (1988).
- Liaaen-Jensen, S. *Acta chem. Scand.* **19**, 1025–1028 (1965).
- Pfennig, N. in *The Photosynthetic Bacteria* (eds Clayton, R. K. & Sistrom, W. R.) 3–18 (Plenum, New York, 1978).
- Brockman, H., Jr *Phil. Trans. R. Soc. Ser. B* **273**, 277–285 (1976).
- Trüper, H. G. & Genovese, S. *Limnol. Oceanogr.* **13**, 225–232 (1968).
- Bergstein, T., Henis, Y., and Cavari, B. Z. *Can. J. Microbiol.* **25**, 999–1007.

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Distribution of two distinct Ca²⁺-ATPase-like proteins and their relationships to the agonist-sensitive calcium store in adrenal chromaffin cells

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MANY cellular functions are regulated by activation of cell-surface receptors that mobilize calcium from internal stores sensitive to inositol 1,4,5-trisphosphate (Ins(1,4,5)P₃) (ref. 1). The nature of these internal calcium stores and their localization in cells is not clear and has been a subject of debate. It was originally suggested^{2–5} that the Ins(1,4,5)P₃-sensitive store is the endoplasmic reticulum, but a new organelle, the calciosome, identified by its possession of the calcium-binding protein, calsequestrin⁶, and a Ca²⁺-ATPase-like protein⁷ of relative molecular mass 100,000 (100K), has been described as a potential Ins(1,4,5)P₃-sensitive calcium store. Direct evidence on whether the calciosome is the Ins(1,4,5)P₃-sensitive store is lacking. Using monoclonal antibodies raised against the Ca²⁺-ATPase of skeletal muscle sarcoplasmic reticulum⁸, we show that bovine adrenal chromaffin cells contain two Ca²⁺-ATPase-like proteins with distinct subcellular distributions. A 100K Ca²⁺-ATPase-like protein is diffusely distributed, whereas a 140K Ca²⁺-ATPase-like protein is restricted to a region in close proximity to the nucleus. In addition, Ins(1,4,5)P₃-generating agonists result in a highly localized rise in cytosolic calcium concentration ([Ca²⁺]_i) initiated in a region close to the nucleus, whereas caffeine results in a rise in [Ca²⁺]_i

throughout the cytoplasm. Our results indicate that chromaffin cells possess two calcium stores with distinct Ca²⁺-ATPases and that the organelle with the 100K Ca²⁺-ATPase is not the Ins(1,4,5)P₃-sensitive store.

Three monoclonal antibodies raised against the Ca²⁺-ATPase of skeletal muscle sarcoplasmic reticulum⁸ were used to identify the distribution of putative calcium stores in adrenal chromaffin cells. The antibodies fortuitously had different reactivities with chromaffin cell polypeptides. In chromaffin cells (Fig. 1), monoclonal antibody 1/2H7 recognized a polypeptide of relative molecular mass 140K, whereas monoclonal Y/1F4 recognized a distinct polypeptide of relative molecular mass 100K. Monoclonal antibody B/3D6 was used as a control as it did not recognize any chromaffin cell polypeptides in immunoblotting

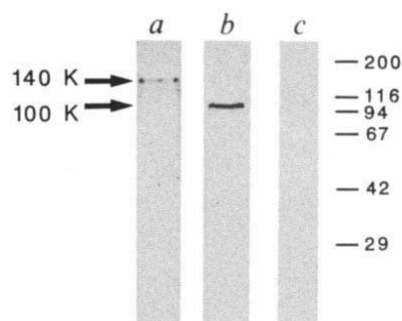


FIG. 1 Characterization of polypeptides recognized by monoclonal antibodies against skeletal muscle Ca²⁺-ATPase in adrenal chromaffin cells. After SDS-PAGE, polypeptides recognized by the antibodies were determined by immunoblotting with a, 1/2H7; b, Y/1F4; and c, B/3D6. On the right are indicated the migration positions of molecular weight standards (in thousands).

METHODS. Chromaffin cells were dissociated from bovine adrenal medulla and grown in 24-well trays at a density of 7×10^5 cells per well as described previously¹⁰. After 24 h in culture the cells were solubilized in 150 µl of SDS-solubilization buffer²⁵ and polypeptides separated by SDS-PAGE on 10% slab gels. After transfer of proteins to nitrocellulose by transverse electrophoresis, the blots were stained with Ponceau S, washed with PBS medium and incubated with 3% non-fat skimmed milk in PBS for 30 min, followed by incubation for 60 min with the monoclonal antibodies diluted (1:250 for 1/2H7, 1:100 for Y/1F4 and 1:250 for B/3D6) in PBS. The blots were then incubated sequentially with anti-mouse biotin (Amersham; diluted 1:100) for 60 min and horseradish peroxidase-streptavidin (Amersham; diluted 1:500) for 30 min in 3% non-fat skimmed milk, 0.5% Tween 20 in PBS, and the reaction developed with diaminobenzidine hydrochloride/H₂O₂.

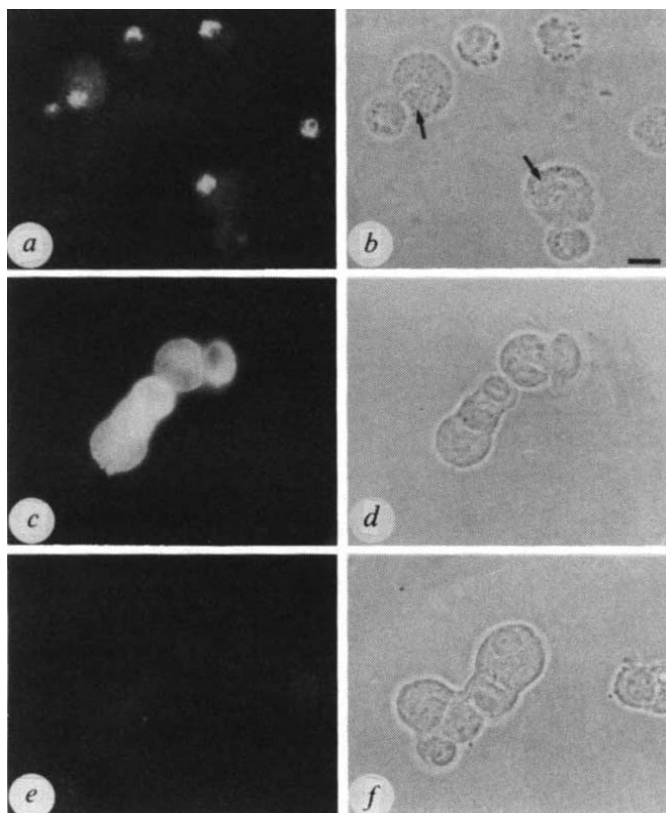


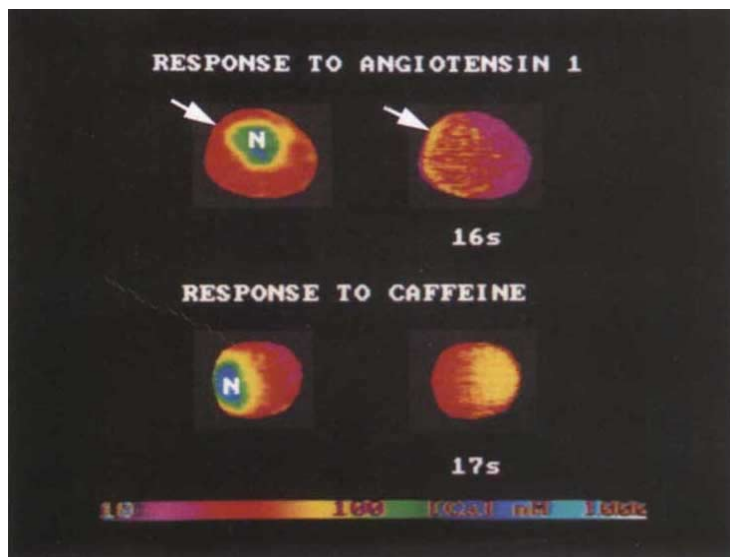
FIG. 2 Immunofluorescence localization of Ca^{2+} -ATPase-like proteins in adrenal chromaffin cells. Chromaffin cells were stained with 1/2H7 (a, b), Y/1F4 (c, d) or B/3D6 (e, f). Fluorescence images are shown in a, c, e and corresponding phase-contrast images in b, d, f. Antibody 1/2H7 gives a localized staining pattern restricted to a region over the nucleus of the cells. In some cases the whole of the nucleus is not stained, it being restricted to a region at the back. By contrast, Y/1F4 gives a diffuse staining pattern over the whole cell and B/3D6 does not stain the cells. Scale bar in b, 10 μm . METHODS. Adrenal chromaffin cells were grown in culture overnight on glass coverslips and fixed in 4% formaldehyde. The cells were washed in PBS and incubated in 0.3% BSA, 0.1% Triton X-100 in PBS (PBT) for 30 min, followed by incubation with the monoclonal antibodies diluted in PBT (1:20 for B/3D6 and Y/1F4, and 1:250 for 1/2H7) for 60 min. The cells were then incubated sequentially with anti-mouse biotin (Amersham; 1:100) for 60 min and Texas-red streptavidin (Amersham 1:50) for 30 min.

and did not give any staining in immunofluorescence on cultured chromaffin cells (Fig. 2). The 140K protein detected by 1/2H7 was restricted in its distribution to the nuclear region of the cells. This staining of the nucleus was not always uniform but was restricted in some cases to the region facing the plasma membrane, on the side opposite the bulk of the cytoplasm (Fig. 2a and b). This staining pattern found with 1/2H7 indicates that the 140K protein is either a component of endoplasmic reticulum (ER) membranes closely associated with the nuclear envelope, or a component of the nuclear envelope itself. Using immunoblotting, we found the 140K protein to be present in a microsomal fraction of adrenal medulla, suggesting that the protein is in the ER (data not shown). By contrast, the 100K protein recognized by Y/1F4 was found to be diffusely distributed in chromaffin cells (Fig. 2c, d). The distribution of the 100K protein was not due to its being a component of the secretory granules (data not shown).

This variation in the distribution of the 140K and 100K proteins was found to correspond to the spatial organization of the calcium signals induced by stimuli that act through different calcium stores. First, the site of the rise in $[\text{Ca}^{2+}]_i$ in response to agonists such as methacholine, angiotensin I and angiotensin II was established in single fura-2-loaded cells by video imaging⁹⁻¹¹ and the nucleus was then located by staining the same cells with ethidium bromide. We had previously shown that rises in $[\text{Ca}^{2+}]_i$ in chromaffin cells in response to $\text{Ins}(1,4,5)\text{P}_3$ -generating agonists, are spatially restricted and are initiated in one pole of the cells^{9,10}. These rises occur even in the absence of external calcium. About 60% of cells showed a polarized rise in $[\text{Ca}^{2+}]_i$, and in these cells, challenged with methacholine ($n=22$), angiotensin I ($n=11$) or angiotensin II ($n=17$), the rise in $[\text{Ca}^{2+}]_i$ originated in a region of the cell between the nucleus and the plasma membrane (Fig. 3a). Thus, the $\text{Ins}(1,4,5)\text{P}_3$ -sensitive calcium store and the 140K Ca^{2+} -ATPase share a similar localization close to the nucleus in chromaffin cells. Recent cell fractionation studies¹² and the immunocytochemical localization of the $\text{Ins}(1,4,5)\text{P}_3$ receptor¹³ provide evidence that a portion of the ER is the $\text{Ins}(1,4,5)\text{P}_3$ -sensitive calcium store. Our observation that the $\text{Ins}(1,4,5)\text{P}_3$ -sensitive store in chromaffin cells lies near the nucleus is consistent with the finding that the ER surrounding the nucleus of cerebellar Purkinje cells stains with an antibody against the $\text{Ins}(1,4,5)\text{P}_3$ receptor¹³. The localization of the mobilizable calcium store next to the nucleus may be important in the regulation of events within the nucleus. In the bovine adrenal chromaffin cell, a rise in $[\text{Ca}^{2+}]_i$ due to release from the internal stores is relatively ineffective in eliciting secretion⁹⁻¹¹ but could regulate

FIG. 3 Localization of the agonist-induced rise in $[\text{Ca}^{2+}]_i$ in the nucleus in chromaffin cells by video-imaging. False-colour images show on the left the location of the nucleus (N) in each cell and on the right the change in $[\text{Ca}^{2+}]_i$ due to 0.3 μM angiotensin I or 10 mM caffeine. In each case the basal $[\text{Ca}^{2+}]_i$ was subtracted from the level after stimulation so that the figures show the change in $[\text{Ca}^{2+}]_i$ from basal levels. The arrows point to the region with the highest rise in $[\text{Ca}^{2+}]_i$ (144 nM above basal; mean basal 60 nM) in response to angiotensin I. The maximum increase in $[\text{Ca}^{2+}]_i$ in response to caffeine in the cell shown was 139 nM above basal level (mean basal 64 nM).

METHODS. Imaging of $[\text{Ca}^{2+}]_i$ in single fura-2-loaded cells was carried out as described previously^{9,10}. Fluorescent images were obtained by alternate excitation at 340 nm or 380 nm (40 ms at each wavelength) using an image-processing system (Imagine, Synoptics Ltd, Cambridge UK) interfaced to a DEC microvax II microcomputer. The ratio image was obtained at video rate and filtered with a time constant of 200 ms. Determination of $[\text{Ca}^{2+}]_i$ was as described by equation 5 of ref. 26. Finally, the cells were stained with 5 mg ml^{-1} ethidium bromide.



other aspects of cell function such as synthesis of biosynthetic enzymes and peptide precursors.

A different pattern of localization of $[Ca^{2+}]_i$ was seen in response to caffeine ($n=31$), which also releases Ca^{2+} from internal stores in chromaffin cells¹⁴. In this case, the rise in $[Ca^{2+}]_i$ was low over the nucleus but occurred throughout the cytoplasm of the cells (Fig. 3b). Similar results were found in the absence of external calcium. It has recently been suggested⁶ that the $Ins(1,4,5)P_3$ -sensitive calcium store is the calciosome. This organelle in hepatocytes and pancreatic acinar cells is stained by a polyclonal antiserum against the skeletal muscle sarcoplasmic reticulum Ca^{2+} -ATPase⁷. This antiserum recognizes a 100K Ca^{2+} -ATPase-like protein in liver¹⁵. The calciosomes are distributed throughout the cytoplasm in many cell types, including adrenal chromaffin cells⁷. In agreement with these findings, we found that Y/1F4, which recognizes a 100K Ca^{2+} -ATPase-like protein, gives a diffuse staining pattern in chromaffin cells. As the rise in $[Ca^{2+}]_i$ in response to $Ins(1,4,5)P_3$ -mobilizing agonists is usually spatially restricted in adrenal chromaffin cells^{9,10}, this indicates that the calciosomes are unlikely to be the $Ins(1,4,5)P_3$ -sensitive calcium store. Instead, the calciosome is likely to be the caffeine-sensitive store because the rise in $[Ca^{2+}]_i$ following treatment with caffeine had a similar general distribution to that found for the 100K Ca^{2+} -ATPase-like protein.

The major finding of this study is that chromaffin cells possess two calcium stores which are spatially distinct and which differ in their sensitivity to $Ins(1,4,5)P_3$ -mobilizing agonists, and possibly in the nature of their Ca^{2+} -ATPase. The possibility of the existence of two separate calcium stores, one sensitive to agonists that generate $Ins(1,4,5)P_3$ and one insensitive, has been inferred for a variety of other cell types^{5,16-20}. In smooth muscle cells^{17,20} and sensory neurons¹⁸, the $Ins(1,4,5)P_3$ -insensitive store is caffeine-sensitive. A similar situation appears to exist in chromaffin cells, where we have shown that the two stores are spatially distinct. The existence of two internal calcium stores has been invoked in one model for the mechanism of calcium oscillations²¹ in response to $Ins(1,4,5)P_3$ -generating agonists²²⁻²⁴. The ability to discriminate between these two stores in chromaffin cells may help in the dissection of the interactions and functional roles of the $Ins(1,4,5)P_3$ -sensitive and $Ins(1,4,5)P_3$ -insensitive stores in calcium signalling. □

Inhibition of noradrenaline release by antibodies to B-50 (GAP-43)

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PROTEIN kinase C (PKC) is believed to have a crucial role in synaptic transmitter release¹⁻⁴ and long-term potentiation⁵⁻⁷. An important substrate of PKC in the brain is the neuron-specific presynaptically localized protein B-50 (also termed GAP-43, F1, pp46 or P-57)⁸⁻¹¹. B-50 has been implicated in the regulation of polyphosphoinositide metabolism^{12,13} and calmodulin binding¹⁴, and in the mechanisms of neurite outgrowth^{15,16}, long-term potentiation¹⁷ and transmitter release¹⁸. It is still unknown, however, whether B-50 (and/or its phosphorylation) is essential to any of these processes. Here we report the results of studies in which antibodies to B-50, which interfere with B-50 phosphorylation, were introduced into rat cortical synaptosomes that were permeabilized with streptolysin-O (SL-O). We found that the release of [³H]noradrenaline, induced by increasing the Ca^{2+} concentration in the buffer, is inhibited completely by the antibodies. These results provide the first demonstration of a causal relationship between the PKC substrate B-50 and the release of neurotransmitter.

Depolarization of intact rat cortical synaptosomes with 10 mM K^+ induced an efflux of noradrenaline (NA) (Fig. 1a, inset). This K^+ -induced efflux was dependent on extracellular Ca^{2+} , confirming that in our system an influx of Ca^{2+} was the intracellular trigger for depolarization-induced neurotransmitter release. To introduce anti-B-50 IgG into synaptosomes, we used a SL-O permeation technique originally developed to study Ca^{2+} -induced vesicular histamine release from mast cells¹⁹. The effect of K^+ depolarization on NA release could be mimicked in SL-O-permeabilized synaptosomes by increasing the Ca^{2+} concentration in the incubation buffer from 10^{-7} to 10^{-5} M (Fig. 1a). To measure basal NA efflux, we used 10^{-7} M Ca^{2+} because (1) this is the resting, intracellular Ca^{2+} concentration in synaptosomes²⁰, and (2) a further reduction of the Ca^{2+} concentration to 10^{-8} M did not reduce NA efflux (see also Fig. 3). As in mast cells, the effect of Ca^{2+} on transmitter release could only be observed in a limited dose range of SL-O (0.6–2.0 IU ml⁻¹; Fig. 1a). The effect of Ca^{2+} was not due to an efflux of cytosolic NA, because Ca^{2+} did not stimulate the SL-O-induced efflux of the cytosolic marker protein, lactate dehydrogenase (Fig. 1b). This confirmed that the Ca^{2+} -dependent part of the SL-O-induced NA efflux was vesicular NA release. From our data we concluded that 0.8 IU ml⁻¹ SL-O is the optimal concentration for permeation of the synaptosomes and for triggering the release of NA with Ca^{2+} .

We tested the effect of anti-B-50 IgG on the extent of endogenous B-50 phosphorylation in synaptosomes, measured with exogenous [γ -³²P]ATP as a phosphate donor. B-50 phosphorylation was only detectable after permeation of synaptosomes with SL-O (Fig. 2), because ATP does not penetrate the intact synaptosome. In control experiments in which H₂O-lysed synaptosomes were used, SL-O had no direct effect on overall kinase activity nor on B-50 phosphorylation. In the SL-O-permeabilized system, anti-B-50 IgG completely inhibited B-50 phosphorylation, whereas control IgG had no effect (Fig. 2). The inhibitory effect of anti-B-50 IgG was highly specific for the phosphorylation of B-50. These results demonstrate that the synaptosomes are sufficiently permeabilized to

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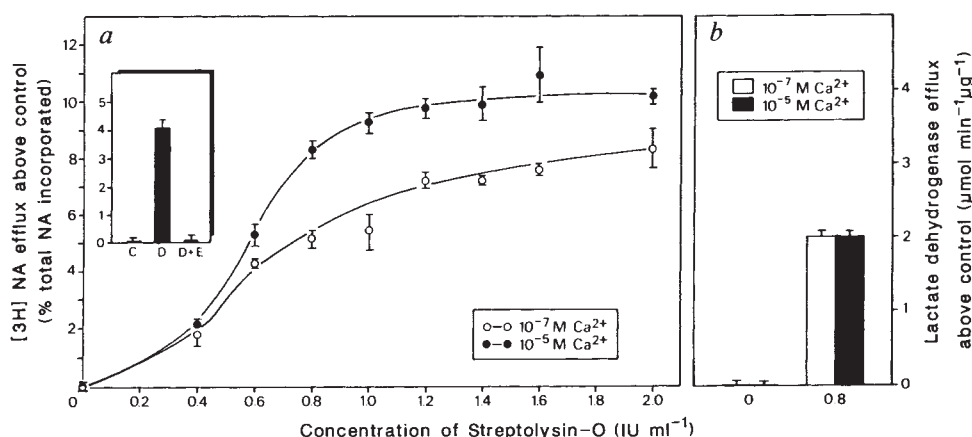
- Berridge, M. J. *A. Rev. Biochem.* **56**, 159–193 (1987).
- Bayerdorffer, E., Streb, H., Eckhardt, L., Haase, W. & Schulz, J. *J. Memb. Biol.* **81**, 69–82 (1984).
- Prentki, M. *et al. Nature* **309**, 562–564 (1984).
- Streb, H., Bayerdorffer, E., Haase, W., Irvine, R. F. & Schultz, J. *J. memb. Biol.* **81**, 241–253 (1984).
- Biden, T. J., Wollheim, C. B. & Schlegel, W. *J. biol. Chem.* **261**, 7223–7229 (1986).
- Volpe, P. *et al. Proc. natn. Acad. Sci. U.S.A.* **85**, 1091–1095 (1988).
- Hashimoto, S. *et al. J. Cell Biol.* **107**, 2523–2531 (1988).
- Colyer, J., Mata, A. M., Lee, A. G. & East, J. M. *Biochem. J.* **262**, 439–447 (1989).
- Cheek, T. R., O'Sullivan, A. J., Moreton, R. B., Berridge, M. J. & Burgoyne, R. D. *FEBS Lett.* **247**, 429–434 (1989).
- O'Sullivan, A. J., Cheek, T. R., Moreton, R. B., Berridge, M. J. & Burgoyne, R. D. *EMBO J.* **8**, 401–411 (1989).
- Cheek, T. R. *J. Cell Sci.* **93**, 211–216 (1989).
- Ghosh, T. K., Mullaney, J. M., Tarazi, F. I. & Gill, D. L. *Nature* **340**, 236–239 (1989).
- Ross, C. A. *et al. Nature* **339**, 468–470 (1989).
- Poisner, A. M. *Proc. Soc. exp. Biol. Med.* **142**, 103–105 (1973).
- Damiani, E., Spamer, C., Heilmann, C., Salvatori, S. & Magreth, A. *J. biol. Chem.* **263**, 340–343 (1988).
- Gill, D. L., Ueda, T., Chueh, S. H. & Noel, M. W. *Nature* **320**, 461–464 (1986).
- Kanaide, H., Shogakiuchi, Y. & Nakamura, M. *FEBS Lett.* **214**, 130–134 (1987).
- Thayer, S. A., Perney, T. M. & Miller, R. J. *J. Neurosci.* **8**, 4089–4097 (1988).
- Busa, W. B., Ferguson, J. E., Joseph, S. K., Williamson, J. R. & Nuclell, R. J. *J. Cell Biol.* **101**, 677–682 (1985).
- Saida, K. & van Breemen, C. *Pflugers Arch.* **397**, 166–167 (1983).
- Woods, N. M., Cuthbertson, K. S. R. & Cobbold, P. H. *Nature* **319**, 600–602 (1986).
- Berridge, M. J. *J. Physiol., Lond.* **403**, 589–599 (1988).
- Berridge, M. J. & Galione, A. *Fedn Proc.* **2**, 3074–3082 (1988).
- Thomas, A. P. *J. biol. Chem.* (in the press).
- Burgoyne, R. D. & Morgan, A. *FEBS Lett.* **245**, 122–126 (1989).
- Grynkiewicz, G., Poenie, M. & Tsien, R. Y. *J. biol. Chem.* **260**, 3440–3450 (1985).

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FIG. 1 *a*, Ca^{2+} -induced efflux of NA in SL-O-permeabilized synaptosomes. Synaptosomes were permeabilized with different concentrations of SL-O at 10^{-7} M ($\circ$) and 10^{-5} M ($\bullet$) free Ca^{2+} . The inset shows Ca^{2+} -dependent K^{+} -induced release of NA in non-permeabilized synaptosomes (C, control; D, K^{+} -depolarized; D+E, K^{+} -depolarized in Ca^{2+} -free buffer). NA efflux above control is expressed as % of total NA incorporated. Control efflux (in the absence of SL-O) was 14.73% and 11.36% for Fig. 1*a* and Fig. 1*a* inset respectively. Data are expressed as mean $\pm$ s.e.m. ($n=3$) *b*, Efflux of the cytosolic marker protein lactate dehydrogenase from permeabilized and non-permeabilized synaptosomes.

Synaptosomes were treated as described below, except that lactate dehydrogenase activity instead of NA was measured in the supernatant. Control efflux (in the absence of SL-O) was $0.5 \mu\text{mol min}^{-1} \mu\text{g}^{-1}$. Lactate dehydrogenase activity was determined according to Bergmeyer and Bernt³². METHODS. Rat cortical synaptosomes were isolated and labelled with NA according to De Langen *et al.*³¹ using the buffer described in ref. 19, supplemented with 2 mM CaCl_2 . They were then washed three times with buffer without CaCl_2 and resuspended at $2 \text{ mg protein ml}^{-1}$. Non-permeabilized synaptosomes (inset): synaptosomes ($20 \mu\text{g protein per } 120 \mu\text{l}$) were



incubated for 5 min at 37°C in buffer with 2 mM CaCl_2 (C), buffer containing 2 mM CaCl_2 and 10 mM K^{+} (D) or in buffer containing 10 mM K^{+} and 1 mM EGTA (D+E). The samples were centrifuged for 30 s at $1,000g$, supernatants were collected and counted in a liquid scintillation counter (Beckman 2000CA). Permeabilized synaptosomes: synaptosomes ($20 \mu\text{g protein per } 120 \mu\text{l}$) were incubated for 5 min at 37°C in buffer containing various concentrations of SL-O (Wellcome, UK), 5 mM ATP, 2 mM MgCl_2 and Ca^{2+} -EGTA buffers with 10^{-7} M or 10^{-5} M free Ca^{2+} (ref. 19). Samples were then processed as described above.

introduce anti-B-50 IgG and that these antibodies specifically inhibit B-50 phosphorylation.

Next we incubated permeabilized synaptosomes with anti-B-50 IgG and applied the Ca^{2+} trigger, and measured NA release. Ca^{2+} -induced release of NA was inhibited by the anti-B-50 IgG in a concentration-dependent way (Fig. 3), whereas control IgG and heat-inactivated anti-B-50 IgG were ineffective at such inhibition. Neither control IgG nor anti-B-50 IgG affected NA efflux at 10^{-8} and 10^{-7} M Ca^{2+} , showing that the antibodies do not influence the Ca^{2+} -independent efflux.

We conclude that B-50 plays a crucial part in the molecular

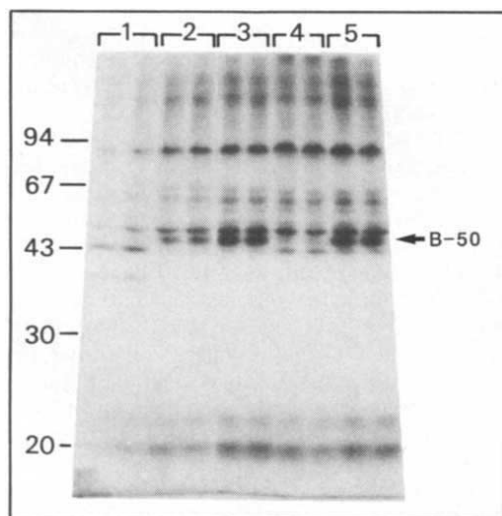


FIG. 2 Autoradiogram showing the effects of anti-B-50 IgG on endogenous protein phosphorylation in SL-O-permeabilized synaptosomes. Synaptosomes were treated with 0 (lanes 1), 0.4 (lanes 2), and 0.8 (lanes 3) IU ml^{-1} SL-O before phosphorylation with $[\gamma\text{-}^{32}\text{P}]\text{ATP}$. The effect of anti-B-50 IgG (lanes 4) or control IgG (lanes 5) was tested in synaptosomes permeabilized with 0.8 IU ml^{-1} SL-O. Position of relative molecular mass markers ($\times 10^3$) shown on left.

METHODS. Synaptosomes ($10 \mu\text{g protein}$) were permeabilized with SL-O for 5 min at 37°C in 30 μl PIPES buffer containing 10^{-5} M Ca^{2+} without ATP. Phosphorylation was started by adding 2 μCi $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ (specific activity 3,000 Ci mmol^{-1} ; Amersham, UK) and carrier ATP to a final concentration of 7.5 μM . After 15 s, phosphorylation was stopped and analysed as described¹⁰. IgG (10 $\mu\text{g per assay}$) were added simultaneously with SL-O. Anti-B-50 IgG was isolated from B-50 antiserum raised in rabbits as described previously³³ by affinity chromatography on a B-50 column³³. Isolated anti-B-50 IgG was extensively dialysed against 1,000 volumes of double distilled water, freeze dried and stored dry at -80°C . On western blots loaded with total synaptosomal proteins, we confirmed the IgG to be specific for B-50. The amount of IgG was determined according to Lowry *et al.*³⁴ with total rabbit IgG (Miles, UK) as reference. Total rabbit IgG was also used as control IgG.

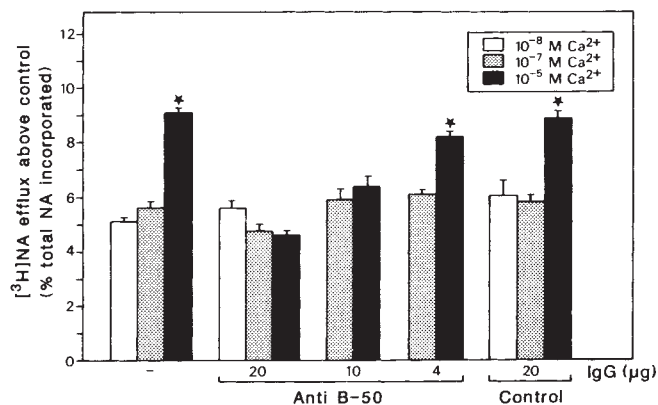


FIG. 3 Effect of anti-B-50 IgG on Ca^{2+} -induced release of NA from SL-O-permeabilized synaptosomes. Synaptosomes were permeabilized with 0.8 IU ml^{-1} SL-O and incubated without IgG (—), with different concentrations of anti-B-50 IgG (anti-B-50), or with control IgG (control) as described in Fig. 2 legend (open bars, 10^{-8} M Ca^{2+} ; dotted bars, 10^{-7} M Ca^{2+} ; filled bars, 10^{-5} M Ca^{2+}). Neither anti-B-50 nor control IgG (20 $\mu\text{g per assay}$) affected NA release in non-permeabilized synaptosomes. Data are expressed as mean $\pm$ s.e.m. (three independent experiments). Identical results were obtained in a separate experiment using a different batch of anti-B-50 IgG. Data were analysed by one-way analysis of variance (ANOVA) with a supplemental Newman-Keuls test. Asterisk denotes group significantly different ($P < 0.05$) from all groups treated with 10^{-7} or 10^{-8} M Ca^{2+} .

mechanism of NA release from rat cerebral-cortex synaptosomes. Because immunocytochemical studies have shown that B-50 is found in synapses throughout the brain^{21,22}, B-50 could be more generally involved in transmitter release. Three lines of evidence suggest that the phosphorylation of B-50 by PKC is essential for stimulus-secretion coupling during transmitter release: (1) phorbol esters that directly activate PKC enhance the release of a variety of neurotransmitters¹⁻⁴; (2) by using an antibody-independent approach, we have previously shown that depolarization-induced neurotransmitter release from non-permeabilized synaptosomes and hippocampal slices is closely correlated with a PKC-mediated increase in B-50 phosphorylation^{18,23}; and (3) here we have shown that anti-B-50 IgG inhibits B-50 phosphorylation as well as Ca²⁺-dependent transmitter release. If B-50 phosphorylation by PKC is indeed involved in the mechanism of transmitter release, then a long-term increase in PKC-mediated B-50 phosphorylation^{7,17} could be one of the mechanisms underlying the increase in the release of glutamate that occurs during long-term potentiation^{5-7,24,25}.

In view of the localization of B-50 at the inner leaflet of the plasma membrane^{21,26}, we suggest that B-50 is involved in the regulation of vesicle fusion with the plasma membrane, a process in which the vesicle-associated protein synapsin I (a substrate of calmodulin-dependent kinases) has also been implicated^{27,28}. But the difference in the localization of phosphorylating enzymes of these two proteins indicates that they have distinct roles in the transmitter release process. It may be that the regulatory role of B-50 in vesicle fusion is not limited to transmitter release, but extends to membrane-fusion processes during neurite outgrowth^{29,30}. It remains to be investigated to what extent calmodulin binding¹⁴ and modulation of phosphatidyl inositol 4-phosphate kinase activity^{12,13}—putative properties of B-50—are also involved in controlling neurotransmitter release. □

Production of antibodies in transgenic plants

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COMPLEMENTARY DNAs derived from a mouse hybridoma messenger RNA were used to transform tobacco leaf segments followed by regeneration of mature plants. Plants expressing single gamma or kappa immunoglobulin chains were crossed to yield progeny in which both chains were expressed simultaneously. A functional antibody accumulated to 1.3% of total leaf protein in plants expressing full-length cDNAs containing leader sequences. Specific binding of the antigen recognized by these antibodies was similar to the hybridoma-derived antibody. Transformants having γ - or κ -chain cDNAs without leader sequences gave poor expression of the proteins. The increased abundance of both γ - and κ -chains in transformants expressing assembled gamma-kappa complexes was not reflected in increased mRNA levels. The results demonstrate that production of immunoglobulins and assembly of functional antibodies occurs very efficiently in tobacco. Assembly of subunits by sexual cross might be a generally applicable method for expression of heterologous multimers in plants.

The source of immunoglobulin mRNAs was a hybridoma cell line expressing a catalytic IgG₁ antibody (6D4) which binds a low molecular weight phosphonate ester (P3) and catalyses the hydrolysis of certain carboxylic esters. Constructs used for immunoglobulin expression in plants consisted of coding-length cDNAs of the 6D4 γ - or κ -chain with or without their leader sequences. These cDNAs were modified to contain terminal *Eco*R1 restriction enzyme digestion sites and were ligated into the constitutive plant expression vector pMON530 (ref. 2) to form pHi101 (kappa, no leader), pHi102 (kappa, leader), pHi201 (gamma, no leader) and pHi202 (gamma, leader). We transformed tobacco plants using *Agrobacterium* containing each of these four plasmids³ and screened leaf extracts from regenerated transformants for the presence of immunoglobulin heavy or light chains by enzyme-linked immunosorbent assay (ELISA)⁴. Transformants expressing individual immunoglobulin chains were then sexually crossed to produce progeny expressing both chains. The results of the ELISA revealed high levels of kappa and gamma chains accumulating in individual plants containing DNA from both pHi102 and pHi202 (Table 1; Fig. 2a). We verified the expression of both heavy and light chains by western blotting (Fig. 1). From the ELISAs, we judged that virtually all the γ - and κ -chains in these plants were assembled into gamma-kappa complexes (Table 1). Western blots provided additional evidence for assembled antibodies in that, under non-reducing conditions, most of the immunoreactive γ - and κ -chains aggregated at a high molecular weight (Fig. 1).

The binding specificity of the assembled gamma-kappa complexes was studied in ELISAs in which a P3-bovine serum albumin conjugate was used as antigen. The antigen binding by antibody derived from plants was equivalent to antigen binding by the 6D4 hybridoma antibody. Incubation of plant extracts or the purified 6D4 antibody with 50 $\mu\text{mol l}^{-1}$ P3 for 3 h at 25 °C before addition to the ELISA eliminated antibody binding to the P3-BSA conjugate, demonstrating that binding was specific for the P3 hapten. Half-maximal inhibition occurred with 10 $\mu\text{mol l}^{-1}$ free P3 for both hybridoma and plant-derived antibodies.

Transformants derived from the leaderless constructs pHi101 and pHi201 contained very low levels of κ - and γ -chains respectively, but Southern and northern blots (Fig. 2) demonstrated the presence of transforming DNA and immunoglobulin transcripts. None of the plants expressing leaderless

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1. Allgaier, C., Von Kügelgen, O. & Hertting, G. *Eur. J. Pharmacol.* **129**, 389-392 (1986).
2. Malenka, R. C., Ayoub, G. S. & Nicoll, R. A. *Brain Res.* **403**, 198-203 (1987).
3. Nichols, R. A., Haycock, J. W., Wang, J. K. T. & Greengard, P. *J. Neurochem.* **48**, 615-621 (1987).
4. Kaczmarek, L. K. *Trends Neurosci.* **10**, 30-34 (1987).
5. Malenka, R. C., Madison, D. V. & Nicoll, R. A. *Nature* **321**, 175-177 (1986).
6. Malinow, R., Madison, D. V. & Tsien, R. W. *Nature* **335**, 820-824 (1988).
7. Akers, R. F., Lovinger, D. M., Colley, P. A., Linden, D. J. & Routtenberg, A. *Science* **231**, 587-589 (1986).
8. Zwiers, H., Schotman, P. & Gispen, W. H. *J. Neurochem.* **34**, 1689-1699 (1980).
9. Aloyo, V. J., Zwiers, H. & Gispen, W. H. *J. Neurochem.* **41**, 649-653 (1983).
10. De Graan, P. N. E., Dekker, L. V., Oestreicher, A. B., Van der Voorn, L. & Gispen, W. H. *J. Neurochem.* **52**, 17-23 (1989).
11. Skene, J. H. P. A. *Rev. Neurosci.* **12**, 127-156 (1989).
12. Jolles, J. et al. *Nature* **286**, 623-625 (1980).
13. Gispen, W. H., Van Dongen, C. J., De Graan, P. N. E., Oestreicher, A. B. & Zwiers, H. In *Inositol and Phosphoinositides* (eds Blaessdale, J. E., Hauser, G. & Eichberg, J. 399-413 (Humana, Clifton, New Jersey 1985).
14. Andreassen, T. J., Luetjje, C. W., Heideman, W. & Storm, D. R. *Biochemistry* **22**, 4615-4618 (1983).
15. Skene, J. H. P. & Willard, M. J. *J. Cell Biol.* **89**, 86-95 (1981).
16. Goslin, K., Schreyer, D. J., Skene, J. H. P. & Banker, G. *Nature* **336**, 672-674 (1988).
17. Lovinger, D. M. et al. *Brain Res.* **343**, 137-143 (1985).
18. Dekker, L. V., De Graan, P. N. E., Versteeg, D. H. G., Oestreicher, A. B. & Gispen, W. H. *J. Neurochem.* **52**, 24-30 (1989).
19. Howell, T. W. & Gomperts, B. D. *Biochim. biophys. Acta* **927**, 177-183 (1987).
20. Verhage, M., Besselsen, E., Lopes Da Silva, F. H. & Ghijsen, W. E. J. M. *J. Neurochem.* **51**, 1667-1674 (1988).
21. Gispen, W. H., Leunissen, J. L. M., Oestreicher, A. B., Verkleij, A. J. & Zwiers, H. *Brain Res.* **328**, 381-385 (1985).
22. Benowitz, L. I., Apostolides, P. J., Perrone-Bizzozero, N., Finklestein, S. P. & Zwiers, H. *J. Neurosci.* **8**, 339-352 (1988).
23. Schrama, L. H. et al. *Soc. Neurosci. Abstr.* **14**, 197.15 (1988).
24. Dolphin, A. C., Errington, M. L. & Bliss, T. V. P. *Nature* **297**, 496-498 (1989).
25. Aniksztejn, L., Roisin, M. P., Amsellam, R. & Ben-Ari, Y. *Neuroscience* **28**, 387-392 (1989).
26. Skene, J. H. P. & Virág, I. *J. Cell Biol.* **108**, 613-624 (1989).
27. Böhler, M. & Greengard, P. *Nature* **326**, 704-707 (1987).
28. Hirokawa, N., Sobue, K., Kanda, K., Harada, A. & Yorifuji, H. *J. Cell Biol.* **108**, 111-126 (1989).
29. van Hooff, C. O. M. et al. *J. Cell Biol.* **108**, 1115-1125 (1989).
30. Zuber, M. X., Goodman, D. W., Karns, L. R. & Fishman, M. C. *Science* **244**, 1193-1195 (1989).
31. De Langen, C. D. J., Hogenboom, F. & Mulder, A. H. *Eur. J. Pharmacol.* **60**, 79-89 (1979).
32. Bergmeyer, H. U. & Bernt, E. In *Methoden der Enzymatischen Analyse* (ed. Bergmeyer, H. U.) 533-538 (Verlag Chemie, Weinheim, 1970).
33. Oestreicher, A. B., van Dongen, C. J., Zwiers, H. & Gispen, W. H. *J. Neurochem.* **41**, 331-340 (1983).
34. Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. *J. Biol. Chem.* **193**, 265-275 (1951).

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immunoglobulin chains contained assembled gamma-kappa complexes (Table 1).

The increased recovery of immunoglobulin epitopes from transformants expressing full-length cDNAs was not reflected in increased mRNA transcript levels. Northern blots (Fig. 2b) comparing pH201 and pH202 transformants, for example, revealed nearly equivalent levels of heavy-chain transcripts, although ELISAs indicated a 40-fold increase in accumulation of heavy-chain protein in the pH202 transformant. Likewise, immunoglobulin mRNA levels in a plant producing large amounts of assembled antibodies were not significantly different from the parental plants that accumulated low levels of immunoglobulin chains (Fig. 2b).

Our results show that individual cDNAs for immunoglobulin κ - and γ -chains can be efficiently expressed in tobacco to form functional antibodies. Assembly of immunoglobulin chains by sexual cross in plants represents a useful alternative to the expression by a single vector of both gamma and kappa cDNAs as in yeast or bacteria⁵⁻⁷, or double transformation with vectors containing individual cDNAs^{8,9}. Potentially, this method is applicable to the assembly of oligomers other than antibodies. The characterization of antibodies produced in plants (glycosy-

lation, processing of leader sequences, cytolocalization and turnover) will be described in a later paper.

In B lymphocytes, immunoglobulin processing and assembly occurs in the endoplasmic reticulum/Golgi in a process that may be promoted by heavy-chain binding proteins present in the endoplasmic reticulum^{10,11}. Plant cells may also have a system for multimer assembly in their endoplasmic reticulum/Golgi that can recognize immunoglobulin chains. Alternatively, assembly may occur spontaneously, given sufficient levels of each chain in the appropriate cellular compartment. Our results demonstrate that plants require a signal sequence for efficient assembly of γ - and κ -subunits. The presence of the mouse leader sequence clearly augments the accumulation of individual chains. This might be the result of an enhanced translation of the immunoglobulin messengers or an increased stability of each protein as a result of subcellular sequestering or secretion. The yield of each chain is increased in plants expressing both gamma and kappa, indicating that assembly of the gamma-kappa complex might enhance stability.

TABLE 1 Expression and assembly of immunoglobulin gamma and kappa chains in tobacco

Accumulation of γ - or κ -chains in transformed plants*			
γ NL 30 $\pm$ 16 (60)	γ L 1,412 $\pm$ 270 (2,400)	γ L(κ L) 3,330 $\pm$ 2,000 (12,800)	γ NL(κ NL) 32 $\pm$ 26 (60)
κ NL 1.4 $\pm$ 1.2 (3.5)	κ L 56 $\pm$ 5 (80)	κ L(γ L) 3,700 $\pm$ 2,300 (12,800)	κ NL(γ NL) 6.5 $\pm$ 5 (20)
Distribution and assembly in crosses†			
	γ only	κ only	$\gamma\kappa$
κ NL $\times$ γ NL	4	6	3 (0% assembly)
κ L $\times$ γ L	3	10	11 (95 $\pm$ 16% assembly)
			Null
			5

* Accumulation of individual gamma and kappa chains (in ng per mg total protein) was estimated by ELISA⁴. Microtitre wells were coated with a goat anti-mouse heavy or light chain-specific IgG (Fisher) in 150 mM NaCl, 20 mM Tris-HCl, pH 8.0 (TBS), followed by blocking with 5% non-fat dry milk in TBS. Plant leaves were homogenized in a mortar and pestle at 4 °C after removal of the midvein. To the supernatant a quarter volume of 5 $\times$ TBS was added, and 50 μ l of 1 in 2 serial dilutions were added to each microtitre well. After 18 h at 4 °C, microtitre wells were washed with distilled water at room temperature. Bound γ - or κ -chains were reacted with goat anti-mouse heavy or light chain-specific antibodies conjugated to horseradish peroxidase for 2 h at 37 °C in TBS, and detected according to the manufacturer's instructions. Control microtitre wells contained extracts from plants transformed with pMON530 vector. Values given as mean ($\pm$ s.d.) are derived from at least two determinations per plant and do not include transformants producing no detectable γ - or κ -chain. At least nine plants were assayed in each category. All values are given as ng per mg of total protein in the extract and are derived from the quantity of purified 6D4 antibody required to give an equivalent colour development in ELISA. Total protein in the extract was determined by the Bio-Rad Coomassie assay. Complementary DNAs containing no leader sequences are referred to as γ NL and κ NL; γ L and κ L refer to cDNAs with leader sequences; $\gamma(\kappa)$ refers to gamma chains in a plant that also expresses κ -chains, and vice versa. Numbers in parentheses are values for plants with the highest levels of accumulation.

† The number of plants expressing γ - or κ -chains among the progeny of a sexual cross. The ELISA for assembly used horseradish peroxidase-conjugated anti- κ -chain-specific antibodies to detect antigen bound to microtitre wells coated with unlabelled anti- γ -chain-specific antibodies, and vice versa. Values derived from these assays were used to calculate the per cent of assembly by comparison with the purified 6D4 antibody. This was determined at least three times for each $\gamma\kappa$ plant. The per cent assembly is expressed in parentheses as the mean $\pm$ s.d.

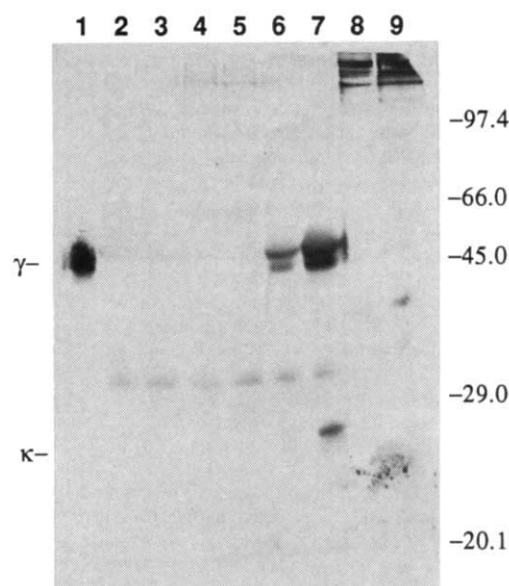


FIG. 1 Western blot of leaf proteins from transgenic tobacco plants expressing immunoglobulin chains. Leaf segments (1 g) from mature plants were homogenized in a mortar and pestle with 1 ml 0.05 M Tris-HCl, pH 7.5, 1 mM phenylmethanesulphonyl fluoride. Extracts were boiled in 4 M urea, 1% SDS, with or without 2 mM dithiothreitol (DTT) as indicated, for 3 min. SDS-PAGE in 10% acrylamide¹⁶ and blotting of the proteins to nitrocellulose¹⁷ were performed as described. Blots were preincubated for 6 h at 4 °C in 20 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.01% Tween 20 (TBST) containing 5% BSA, and 0.5% non-fat dried milk before the addition of antibodies. A biotinylated goat anti-mouse whole IgG antibody (Cappel), diluted 1:500 in TBST was used to probe the blots at 4 °C for 24 h. A variety of commercially available antibodies (anti-mouse IgGs) were used in other experiments with similar results. Antibody binding was visualized after binding of streptavidin-conjugated alkaline phosphatase (25 °C, for 2 h) by incubation in 300 μ g ml⁻¹ nitroblue tetrazolium and 150 μ g ml⁻¹ 5-bromo-4-chloro-3-indolyl phosphate. In lanes 1-7, 40 μ l of each extract containing DTT; lanes 8 and 9, 40 μ l extract without DTT. Lane 1, 100 ng purified antibody from the 6D4 hybridoma; lane 2, 15 μ g wild-type plant-extract protein; lane 3, 15 μ g protein from a plant transformed with truncated κ -chain cDNA (pHi101) containing no leader sequence; lane 4, 15 μ g from plant transformed with truncated γ -chain cDNA (pHi201); lane 5, 15 μ g from a full-length kappa cDNA transformant (pHi102); lane 6, 15 μ g from a full-length γ -chain cDNA transformant (pHi202); lane 7, 15 μ g from an F1 plant derived from the cross between a kappa and a gamma producer; lane 8, 100 ng 6D4 antibody (no DTT); lane 9, same as lane 7, except no DTT. Gamma and kappa on the left refer to the positions of the 6D4 heavy and light chains; positions of molecular weight (given in thousands) markers are shown on the right. By ELISA, extracts in lanes 3-5 contained very low levels of κ - or γ -chains (<0.008% of total protein, Table 1), whereas extracts in lanes 6, 7 and 9 contained 0.24, 1.3 and 1.3% immunoglobulin respectively.

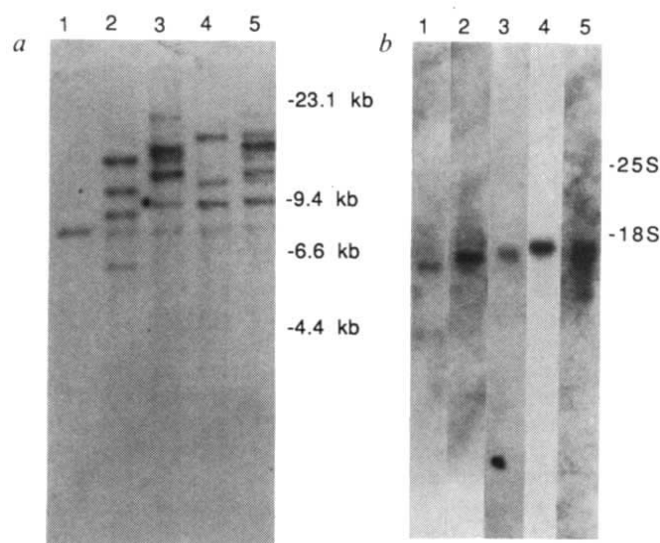


FIG. 2 Southern and northern blots of leaf DNA and RNA from transgenic plants expressing immunoglobulin cDNAs. *a*, DNA was extracted from 1 g mature leaf tissue after freezing the fresh segments in liquid N_2 . Homogenization into urea mix¹⁸ was in a mortar and pestle. The homogenate was extracted with phenol:CHCl₃ (1:1) and the nucleic acids precipitated by addition of one volume of isopropyl alcohol. The resuspended DNA (20 µg each) was cut with *Hind*III and Southern-blotted as described¹⁹. The probe used for both Southern and northern blots was ³²P-labelled pMON530 plasmid² containing either a kappa cDNA or a gamma cDNA. Both cDNAs were used in the hybridization shown. Lane 1, DNA from a transformant expressing a light-chain cDNA without a leader sequence (pHi101); lane 2, DNA from a heavy-chain cDNA transformant, no leader (pHi201); lane 3, DNA from a transformant expressing full-length light chain with leader (pHi102); lane 4, DNA from a transformant expressing heavy chain with leader (pHi202); lane 5, DNA from an F₁ plant derived from a cross between plants expressing full-length gamma or kappa cDNAs (pHi102 × pHi202). *b*, Extraction of RNA from 1 g fresh leaf tissue was by homogenization in 10 ml 0.1 M Tris-HCl, pH 9.0, and 10 ml phenol saturated with this buffer, using a Polytron at high speed. Nucleic acids were precipitated by addition of one tenth volume 3.0 M sodium acetate, pH 5.0, and 1.5 volumes isopropyl alcohol. Resuspended RNA was electrophoresed in gels containing formaldehyde and northern blotted onto nylon membranes (Amersham) as described¹⁹. Lane 1, RNA from a transformant expressing a light-chain cDNA without a leader sequence (pHi101); lane 2, RNA from a heavy-chain cDNA transformant, no leader (pHi201); lane 3, RNA from a transformant expressing full-length light chain with leader (pHi102); lane 4, RNA from a transformant expressing heavy chain with leader (pHi202); lane 5, RNA from an F₁ plant derived from a cross between plant expressing full-length gamma or kappa cDNAs (pHi102 × pHi202). Total RNA (20 µg) was loaded in each lane. Lanes from separate hybridizations were aligned with respect to the 18S (1,900 base pairs) and 25S (3,700 base pairs) ribosomal RNA bands on the blots, detected by methylene-blue staining.

Expression of functional antibodies from transcripts that do not contain signal sequences may require modifications to yield alternative antigen-binding structures (such as single-chain antigen-binding proteins^{6,7}) that do not need to be assembled. Thus binding of constituents of metabolic pathways involved in morphogenesis, stress responses or plant-pathogen interactions could be used to further our understanding of these processes in a way analogous to the blocking by microinjected antibodies of specific protein functions in mammalian cells^{12,13}.

As large macromolecules such as protein multimers do not pass through plant cell walls^{14,15}, the binding by antibodies of small organic molecules (toxins, herbicides, plant hormones, organic chelates, for example) that are permeable to the cell wall might result in the net uptake and retention of these molecules in the plant. Accumulation of functioning antibodies may provide new options for the recovery of an array of environmental contaminants, as well as other biologically significant organics. Catalytic processing of small molecules within cell

wall boundaries by antibodies may also become a generalized strategy for the elimination or modification of permeable organic compounds and could introduce new catalytic properties into existing metabolic pathways. □

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1. Tramontano, A., Janda, K. D. & Lerner, R. A. *Science* **234**, 1566-1570 (1987).
2. Rogers, S. G., Klee, H. J., Horsch, R. B. & Fraley, R. T. *Meth. Enzym.* **153**, 253-277 (1987).
3. Horsch, R. B. *et al.* *Science* **227**, 1229-1231 (1985).
4. Engvall, E. & Perlmann, P. *J. Immun.* **109**, 129-135 (1972).
5. Carlson, J. R. *Mol. cell. Biol.* **8**, 2638-2646 (1985).
6. Huston, J. S. *et al.* *Proc. natn. Acad. Sci. U.S.A.* **85**, 5879-5883 (1988).
7. Bird, R. E. *et al.* *Science* **242**, 423-426 (1988).
8. Wood, C. R. *et al.* *Nature* **314**, 446-449 (1985).
9. Horwitz, A. H., Chang, C. P., Better, M., Hellstrom, K. E. & Robinson, R. R. *Proc. natn. Acad. Sci. U.S.A.* **85**, 8678-8682 (1988).
10. Tartakoff, A. & Vassalli, P. *J. Cell Biol.* **83**, 284-299 (1979).
11. Munro, S. & Pelham, H. R. B. *Cell* **46**, 291-300 (1986).
12. Lamb, N. J. C. *et al.* *J. Cell Biol.* **106**, 1955-1971 (1988).
13. Riabowol, K. T., Vosatka, R. J., Ziff, E. B., Lamb, N. J. & Feramisco, J. R. *Mol. cell. Biol.* **8**, 1670-1676 (1988).
14. Milburn, J. A. *Water Flow in Plants* Longman, (London, 1979).
15. Carpita, N., Sabularse, D., Montezinos, D. & Delmer, D. P. *Science* **205**, 1144-1147 (1979).
16. Chua, N.-H. *Meth. Enzym.* **69**, 434-446 (1980).
17. Harlow, E. & Lane, D. *Antibodies: A Laboratory Manual* (Cold Spring Harbor Laboratory, New York, 1988).
18. Shure, M., Wessler, S. & Fedoroff, N. *Cell* **35**, 225-233 (1983).
19. Maniatis, T., Fritsch, E. F. & Sambrook, J. *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Laboratory, New York, 1982).

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Leu-8/TQ1 is the human equivalent of the Mel-14 lymph node homing receptor

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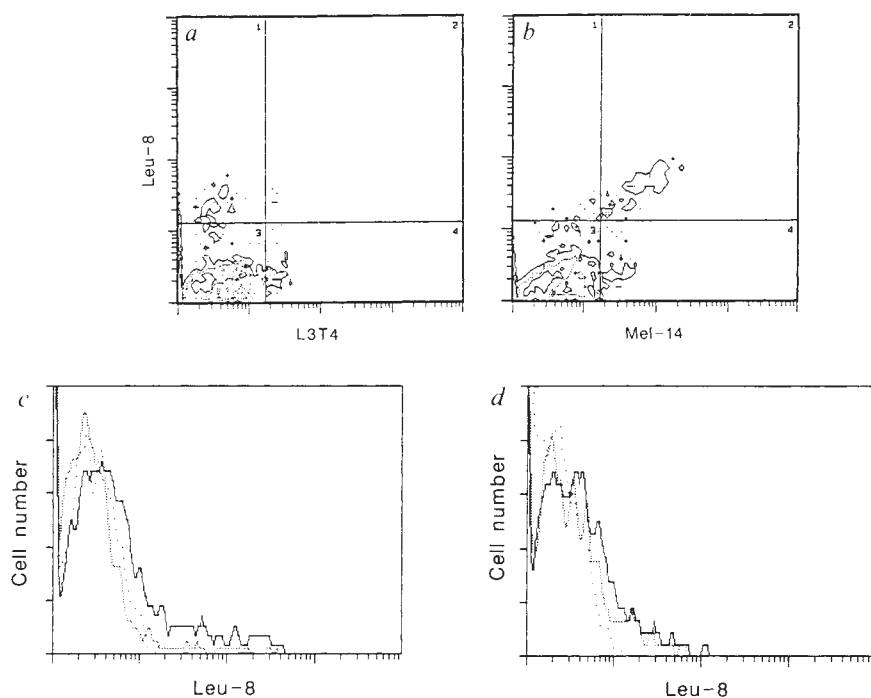
THE human pan-leukocyte antigen Leu-8 has attracted wide interest because its presence or absence identifies suppressor-inducer and helper-inducer CD4⁺ T-lymphocyte subsets respectively. We report here that Leu-8 is the human homologue of the mouse Mel-14 homing receptor, a molecule that promotes the initial adhesion of blood-borne lymphocytes to the specialized post-capillary endothelium of peripheral lymph nodes. We also show that Leu-8 can adopt both conventional and phospholipid anchored forms, a finding that may have relevance in the context of antigen shedding following activation or homing. The assignment of lymphocytes to different functional classes based on lymph node homing potential may represent a more general association between lymphocyte function and tissue distribution.

Two complementary DNA clones encoding Leu-8 determinants were isolated from a human T-cell library by methods previously described^{1,2}. DNA sequence analysis showed that the longer insert of the two contains 2,350 residues, whereas the shorter lacks 436 internal residues but is otherwise identical (Fig. 1). The predicted polypeptide sequences were found to diverge at their C-termini.

The protein encoded by the larger insert bears a strongly hydrophobic putative membrane spanning domain near its C terminus, followed by several positively charged residues resembling a cytoplasmic anchor sequence. The protein is closely related to the recently described murine Mel-14 homing receptor^{3,4} (Fig. 1) and the corresponding cDNA sequence shares

FIG. 2 Flow cytometric analysis of COS cells transfected with Leu-8 cDNA clones. *a, b*, Two-colour analysis with a phycoerythrin-conjugated anti-Leu-8 antibody (Leu-8 PE) and *a*, rat hybridoma L3T4 (control) ascites followed by fluorescein conjugated goat anti-rat IgG (fluorescein isothiocyanate (FITC) goat anti-rat IgG) or *b*, rat hybridoma Mel-14 ascites followed by FITC goat anti-rat IgG. *c, d*, Sensitivity of Leu-8 antigen to release by phosphatidylinositol-specific phospholipase C. COS cells expressing the shorter Leu-8 cDNA clone, *c*, or the longer clone *d*, were incubated with anti-Leu-8 FITC after incubation with phosphatidylinositol-specific phospholipase C or buffer alone at 37 °C for 1 h. In *c* and *d*, the histogram of CD16 (control) transfected COS cells stained with labelled anti-Leu-8 is represented by a sparsely dotted line, the histogram of Leu-8 transfected cells stained with labelled anti-Leu-8 is represented by a solid line, and the histogram of Leu-8 transfected cells treated with phospholipase C and similarly stained is represented by a broken line.

METHODS. For two-colour analysis, COS cells were trypsinized 12–24 h after DEAE-Dextran transfection and passaged in a ratio of 1:2. Post-transfection (60–72 h), the cells were collected by incubation in PBS medium plus 0.5 mM EDTA (PBSE), collected by centrifugation, and resuspended in RPMI medium containing 10% human plasma. L3T4 or Mel-14 ascites was added at 1:50 dilution, and the cells were incubated on ice for 30–40 min, and centrifuged through PBS, 2% Ficoll, 1 mg ml⁻¹ BSA, 0.04% sodium azide (PFBA). The cells were resuspended in PBSE with 20% skim milk containing affinity purified FITC conjugated goat anti-rat IgG (Chemicon) at 50 µg ml⁻¹. After 20 min on ice, the cells were diluted with PBS plus 0.5 mM EDTA, and centrifuged through a two-step gradient of PFBA (0.25 ml) over PBS with 4% formaldehyde (2.5 ml). The cells were allowed to fix for 15 min, then the supernatant was aspirated, and the pellet and cells adhering to the walls of the tube were



resuspended in IMD medium containing 0.02% Nonidet P-40 detergent, and recentrifuged. Anti-Leu-8 PE was added, the cells were incubated on ice for 30 min and then centrifuged through PBS, with 4% formaldehyde and 0.05% Tween-20, and stored for cytometry. For single-colour analysis, the primary antibody was added to the cells in PBS with 0.02% NaN₃ and 5% fetal bovine serum and incubated for 1 h on ice. Cells were washed as for the two-colour analyses. Incubation with phosphatidylinositol-specific phospholipase C was for 1 h at 37 °C in 2.6% glycerol and PBS at a ratio of 9:1.

single copy gene (Fig. 3). RNA blot hybridization revealed a major transcript of ~2.4 kilobases (kb) present in peripheral blood mononuclear cells, tonsillar B cells, and several lymphocytic cell lines; and a minor transcript of 2.0 kb, present in peripheral blood mononuclear cells, and the Jurkat and HSB-2 leukaemic T-cell lines (Fig. 3, and data not shown). Reduced expression of the RNA was detected in peripheral blood lymphocytes activated with phorbol ester plus either phytohaemagglutinin or concanavalin A, whereas treatment with phorbol ester alone induced transcript expression (Fig. 3). Tonsillar B cells activated by anti-IgM antibody, phorbol diester, anti-CD20 monoclonal antibody, G28-8 monoclonal antibody, or gamma interferon showed little change in transcript abundance (Fig. 3).

Western blot analysis allowed at least seven species of similar molecular weight to be visualized from extracts of Leu-8-transfected COS cells (Fig. 3). Extracts prepared from the human T-cell leukaemia line Jurkat, which expresses Leu-8 transcripts and surface antigen (data not shown), showed at least two closely spaced bands with a relative molecular mass of ~76,000 (76 K), the size of the largest forms seen in COS cells.

Twelve hours after activation by phorbol ester plus phytohaemagglutinin, Leu-8 expression by peripheral blood lymphocytes is diminished, whereas Leu-8 transcripts are more prevalent (Fig. 4). At 24 h post-treatment, both surface and transcript expression are substantially reduced (Fig. 4). Thus, surface Leu-8 is lost more rapidly than predicted by RNA turnover, possibly by shedding of a phosphatidylinositol-linked form¹². Leu-8 loss on activation is consistent with serological studies showing that Leu-8 is a marker of resting lymphocytes in peripheral lymph nodes¹³.

The tissue distributions of Leu-8 (refs 14–20) and Mel-14 antigen^{9,23} are similar, with one exception^{24,25}. Although Leu-8

is found on almost all medullary and scattered cortical thymocytes²⁶, histological studies have shown Mel-14 reactivity only on scattered cortical cells²⁷. On this basis it has been proposed that murine cortical thymocytes are mature²⁷; flow cytometry, however, has shown that most mature thymic cells are Mel-14⁺ (ref. 28). The present data support the view that the failure to detect Mel-14 reactivity on thymic medullary cells *in situ* is an unusual histological artefact²⁸.

Among human peripheral blood T cells, the CD4⁺ Leu-8/TQ1⁻ subset bears almost all of the cells providing help for B cell IgM and IgG synthesis^{8,14}, whereas CD4⁺ Leu-8⁺ cells directly inhibit pokeweed mitogen-induced IgG synthesis²⁹. Because T cells that promote immunoglobulin synthesis might be expected to target to, or reside in, peripheral nodes, the finding that CD4⁺ Leu-8⁻ cells provide B-cell help is unexpected. One explanation may be that this subset lacks Leu-8 because it consists of activated cells which have exited the nodes into the peripheral circulation. Consistent with this, the mitotically dormant paracortical and interfollicular areas of peripheral nodes bear Leu-8⁺ T cells, whereas the actively proliferating B-cell germinal centres contain scattered Leu-8⁻ T cells²⁶. Another explanation for why CD4⁺ Leu-8⁻ cells provide B-cell help may be that the peripheral pool of cells providing help for immunoglobulin synthesis mainly arises (under normal physiological circumstances) from gut lymphatic structures, where chronic antigenic stimulation ensures a constant supply of activated T cells.

Because both Leu-8⁺ and Leu-8⁻ cells can be found among CD4⁺ cells proliferating in response to a recall antigen, Leu-8 antigen loss following activation may be reversible on at least a subset of cells, and some memory cells may be Leu-8⁺ (ref. 30). The CD4⁺ Leu-8⁻ population contains all of the cytotoxic

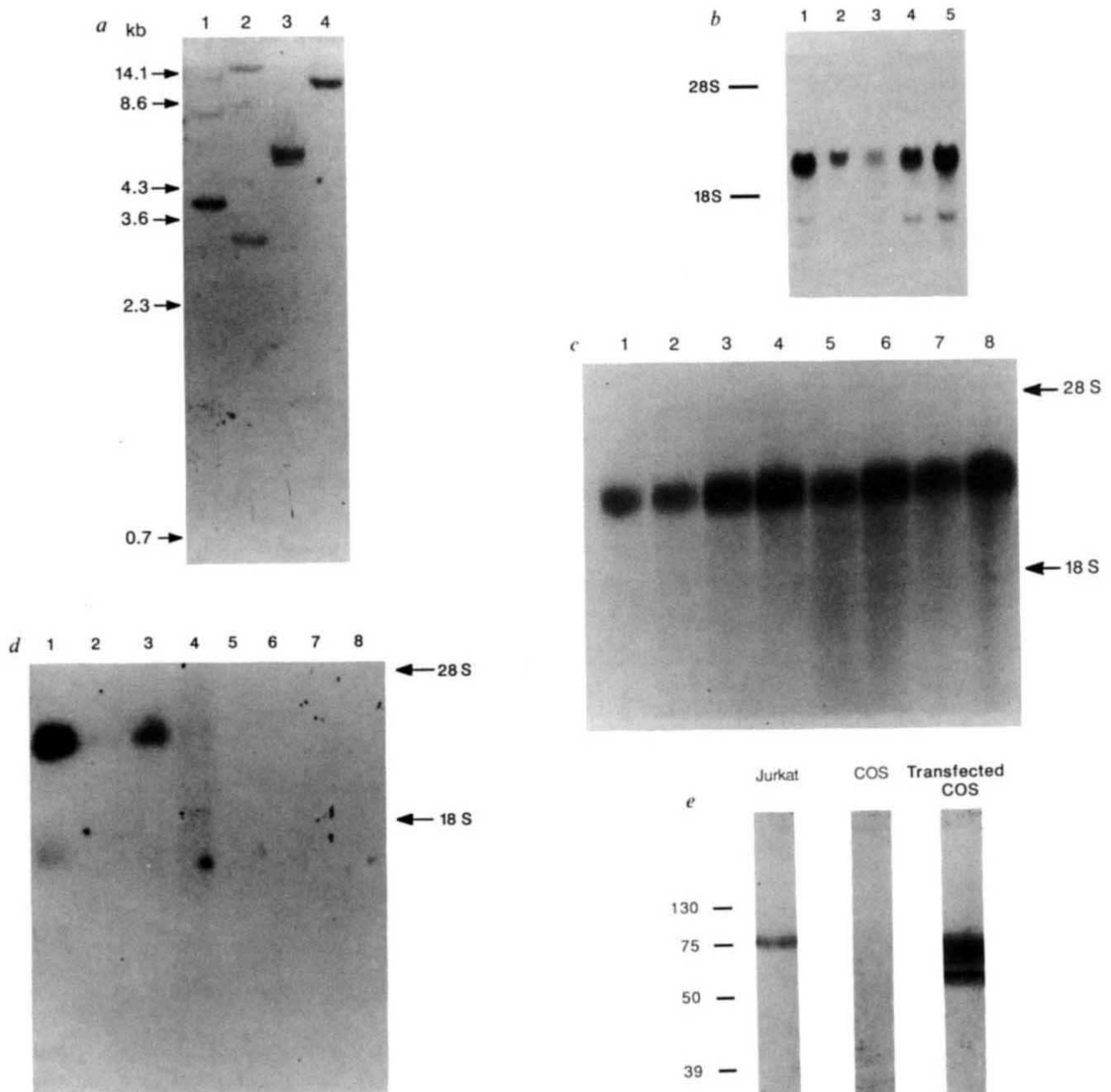


FIG. 3 Leu-8 protein and nucleic-acid analyses. *a*, DNA blot analysis of human DNA digested with *Bam*HI (lane 1), *Eco*RI (lane 2), *Hind*III (lane 3) and *Pst*I (lane 4) using a single-stranded Leu-8 probe. *b–e*, RNA blot analysis of total human RNA with a Leu-8 probe. *b*, Effect of activation on Leu-8 RNA expression by peripheral blood lymphocytes: lane 1, resting lymphocytes; lane 2, lymphocytes stimulated with $5 \mu\text{g ml}^{-1}$ phytohaemagglutinin plus 1 ng ml^{-1} phorbol myristate acetate (PMA); lane 3, lymphocytes stimulated with $5 \mu\text{g ml}^{-1}$ concanavalin A plus 1 ng ml^{-1} PMA; lane 4, lymphocytes stimulated with pokeweed mitogen (GIBCO, 1:100); lane 5, lymphocytes stimulated with 1 ng ml^{-1} PMA. *c*, RNA from resting and stimulated B cells: lane 1, B cell-enriched peripheral blood lymphocytes stimulated with $1 \mu\text{g ml}^{-1}$ γ -interferon (Hoechst); lane 2, unstimulated B cell-enriched resting peripheral blood lymphocytes; lane 3, purified tonsillar B cells stimulated with $5 \mu\text{g ml}^{-1}$ G28-8; lane 4, purified tonsillar B cells stimulated with $5 \mu\text{g ml}^{-1}$ anti-CD20 antibody 1F5; lane 5, resting purified tonsillar B cells; lane 6, purified tonsillar B cells stimulated with $1 \mu\text{g ml}^{-1}$ γ -interferon; lane 7, purified tonsillar B cells stimulated with $50 \mu\text{g ml}^{-1}$ anti-IgM antibody; lane 8, purified tonsillar B cells stimulated with $50 \mu\text{g ml}^{-1}$ PMA; lane 9, U937; lane 10, U937 stimulated with $50 \mu\text{g ml}^{-1}$ PMA; lane 11, THP-1. *d*, Protein blot transfer analysis of Leu-8. Jurkat (T cell leukaemia) cells, COS cells, or COS cells

transfected with the longer Leu-8 clone were solubilized in a lysis buffer containing 1% NP-40 and fractionated on 10% SDS polyacrylamide gels. The fractionated proteins were transferred to nitrocellulose electrophoretically. The filter replica was blocked with gelatin (1% in Tris-buffered saline), treated with anti-Leu-8 ascites (1:10,000), washed, and incubated with goat anti-mouse IgG antibody coupled to alkaline phosphatase (Bio-Rad, 1:1000). Bands were visualized by incubation with nitroblue tetrazolium-bromochloroindolyl phosphate (Bio-Rad).

METHODS. DNA and RNA blot hybridizations were carried out as previously described^{1,2}. Tonsillar B cells were prepared by teasing the tissue in sterile phosphate-buffered saline. Tonsillar or peripheral blood mononuclear cells were washed several times and T cells were removed by incubating with anti-CD3 monoclonal antibody at 4 °C followed by rabbit complement (Pel-Freez) at 37 °C for 45 min in serum-free IMD medium (GIBCO). Monocytes were removed by adherence to plastic dishes, twice for 30 min. 80–85% of the resulting cells were sIgM-positive.

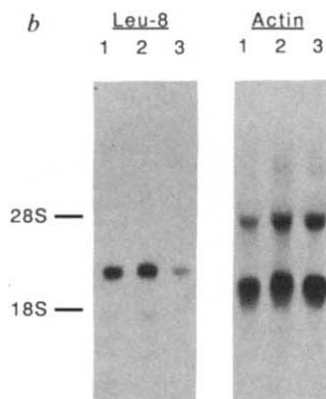
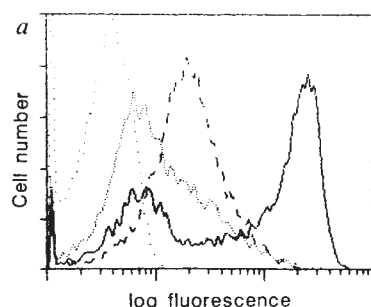


FIG. 4 Leu-8 RNA and surface expression following activation. Peripheral blood lymphocytes were stimulated for the times indicated with $5 \mu\text{g ml}^{-1}$ phytohaemagglutinin (PHA) and 1 ng ml^{-1} PMA. *a*, Flow cytometric analysis of Leu-8 antigen on cells exposed to 0, 12 and 24 h of stimulation. —, Untreated; ---, PHA + PMA (12 h); ····, PHA + PMA (24 h); - · - ·, no stain. *b*, RNA blot analysis of the cells shown in *a*, with a Leu-8 cDNA probe. Lane 1, no treatment; lane 2, stimulated for 12 h; lane 3, stimulated for 24 h. *c*, Hybridization of the blot in *b* with an actin cDNA probe.

(class II restricted) effector cells, whereas only the CD4^+ Leu-8⁺ population proliferates in long-term culture without exogenous cytokine, indicating that this population provides autocrine T-cell help³⁰. As the Leu-8 phenotype of these populations is stable on continued culture *in vitro*³⁰, the CD4^+ Leu-8⁺ cytotoxic/immunoglobulin helper cells may be destined to invade the mucosal lymphatics or extralymphoid sites of inflammation, whereas the CD4^+ Leu-8⁺ autocrine helper cells recirculate through peripheral nodes. □

24. Reichert, R. A., Gallatin, W. M., Weissman, I. L. & Butcher, E. C. *J. exp. Med.* **157**, 813–827 (1983).
25. Kraal, G., Hardy, R. R., Gallatin, W. M., Weissman, I. L. & Butcher, E. C. *Eur. J. Immun.* **16**, 829–834 (1986).
26. Hsu, S.-M. & Jaffe, E. S. *Am. J. Pathol.* **121**, 69–78 (1985).
27. Reichert, R. A., Gallatin, W. M., Butcher, E. C. & Weissman, I. L. *Cell* **38**, 89–99 (1984).
28. Shortman, K., Wilson, K., Van Ewijk, W. & Scollay, R. *J. Immun.* **138**, 342–351 (1987).
29. Kanof, M. E., Strober, W. & James, S. P. *J. Immun.* **139**, 49–54 (1987).
30. Takada, S., Koide, J. & Engleman, E. G. *J. Immun.* **142**, 3038–3044 (1989).

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Interleukin-2-dependent autocrine proliferation in T-cell development

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ACTIVATED T lymphocytes proliferate in response to interleukin-2 (IL-2), which binds to a specific high-affinity receptor (IL-2R)^{1–3}. This consists of at least two noncovalently linked polypeptides, p55/IL-2R α (Tac)⁴ and p75/IL-2R β ^{5–7}. Both molecules bind IL-2 independently, with low and intermediate affinity respectively^{8–10}, but only IL-2R β is thought to mediate IL-2 signal transduction^{8,11–13}. Although IL-2R β seems to be constitutively expressed on resting T lymphocytes^{9,14}, the growth of these T cells is specifically induced by antigenic triggering by the T-cell receptor (TCR), which then results in the transcription of both IL-2 and IL-2R α genes^{12,15}. By contrast, activation of the IL-2/IL-2R pathway in the thymus seems to precede the appearance of the TCR, as IL-2R α is expressed on T-cell precursors lacking TCR^{16–21}. The basis for IL-2R expression by immature thymocytes, however, remains largely unknown. We show here that IL-2R α -negative T-cell precursors constitutively express IL-2R β and produce their own IL-2. The IL-2/IL-2R β interaction on these cells induces the expression of IL-2R α , leading to high-affinity IL-2R display and cellular proliferation. We suggest that this IL-2-dependent autocrine pathway of growth stimulation plays a key role in the intrathymic development of mature T cells.

Adult human thymocytes expressing the CD7 T-cell antigen, but lacking the CD1, CD2, CD3, CD4 and CD8 molecules (pro-T cells), comprise the precursors of IL-2R α ⁺ thymocytes and mature T cells^{21,22}. Although very few pro-T cells express IL-2R α (<1% Tac⁺; Fig. 1*a*), Scatchard analyses of ¹²⁵I-labelled IL-2 binding indicates that there are ~3,250 IL-2 binding sites per cell (assuming a uniform distribution of sites) (Fig. 2*a*). A large majority (3,000 sites per cell) bound IL-2 with the 'intermediate' affinity characteristic of IL-2R β ($K_d \sim 1.9 \text{ nM}$)^{7,8}, whereas only 250 high-affinity receptors per cell ($K_d \sim 26 \text{ pM}$) were detected. Parallel studies conducted in the presence of saturating concentrations (500-fold molar excess) of anti-IL-2R α monoclonal antibody, showed that pro-T cells displayed 2,320 sites per cell ($K_d \sim 850 \text{ pM}$), other than IL-2R α (Fig. 2*b*). This suggests that the IL-2R β intermediate-affinity receptor is the chief IL-2-binding component expressed on pro-T cells. By contrast, an almost threefold lower number of IL-2R β was detected in highly purified, resting peripheral T cells (960 sites per cell, $K_d \sim 820 \text{ pM}$) (Fig. 2*c*).

According to these data, freshly isolated pro-T cells contained virtually no detectable IL-2R α messenger RNA (Fig. 3*a*). Incubation of pro-T cells with doses of recombinant IL-2 (rIL-2) favouring intermediate-affinity binding (1 nM), however, resulted in a rapid induction of IL-2R α gene transcription,

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1. Seed, B. & Aruffo, A. *Proc. natn. Acad. Sci. U.S.A.* **84**, 3365–3369 (1987).
2. Aruffo, A. & Seed, B. *Proc. natn. Acad. Sci. U.S.A.* **84**, 8573–8577 (1987).
3. Lasky, L. A. *et al. Cell* **56**, 1045–1055 (1989).
4. Siegelman, M. H., van de Rijn, M. & Weissman, I. L. *Science* **243**, 1165–1172 (1989).
5. Bowen, B., Nguyen, T. & Lasky, L. J. *Cell Biol.* **109**, 421–427 (1989).
6. Siegelman, M. H. & Weissman, I. L. *Proc. natn. Acad. Sci. U.S.A.* **86**, 5562–5566 (1989).
7. Tedder, T. F. *et al. J. exp. Med.* **170**, 123–133 (1989).
8. Reinherz, E. L. *et al. J. Immun.* **128**, 463–468 (1982).
9. Gallatin, W. M., Weissman, I. L. & Butcher, E. C. *Nature* **304**, 30–34 (1983).
10. Siegelman, M. *et al. Science* **231**, 823–829 (1986).
11. St. John, T. *et al. Science* **231**, 845–850 (1986).
12. Ferguson, M. A. J. & Williams, A. F. *Rev. Biochem.* **57**, 285–320 (1988).
13. Poletti, A., Manconi, R. & De Paoli, P. *Hum. Pathol.* **19**, 1001–1007 (1988).
14. Gatenby, P. A., Kansas, G. S., Xian, C. Y., Evans, R. L. & Engleman, E. G. *J. Immun.* **129**, 1997–2000 (1982).
15. Lanier, L. A. *et al. Immun. Rev.* **74**, 143–160 (1983).
16. Lanier, L. L. & Loken, M. R. *J. Immun.* **132**, 151–156 (1984).
17. Kelly, J., O'Farrelly, C., O'Mahoney, C., Weir, D. G. & Feighery, C. *Clin. exp. Immun.* **68**, 177–188 (1987).
18. James, S. P., Fiocchi, C., Graeff, A. S. & Strober, W. *Gastroenterology* **91**, 1483–1489 (1986).
19. Kansas, G. S., Wood, G. S., Fishwild, D. M. & Engleman, E. G. *J. Immun.* **134**, 2995–3002 (1985).
20. Kansas, G. S., Wood, G. S. & Engleman, E. G. *J. Immun.* **134**, 3003–3006 (1985).
21. Lewinson, D. M., Bargatze, R. F. & Butcher, E. C. *J. Immun.* **138**, 4313–4321 (1987).
22. Lefrançois, L. *J. Immun.* **138**, 3375–3384 (1987).
23. Schmitz, M., Nunez, D. & Butcher, E. C. *Gastroenterology* **94**, 576–581 (1988).

which reached maximal levels after eight days (similar to those observed in mitogen-activated peripheral T cells; Fig. 3a). This induction led to the expression of IL-2R α molecules on most cultured pro-T cells (86%; Fig. 1b). A similar proportion of IL-2R α ⁺ cells (with a reduced fluorescence intensity, probably secondary to the antibody treatment) was observed when a 300-fold molar excess of anti-IL-2R α monoclonal antibody was added to the cultures (81%; Fig. 1c). Binding of IL-2 to individual IL-2R β molecules may, therefore, be an effective signal for the induction of IL-2R α on T-cell precursors. The observation that expression in both culture conditions of IL-2R α was detectable on 5 to 30% of cells after only 24 h of culture, when the cellular recovery was ~70%, formally rules out overgrowth of a minor contaminating subset of IL-2R α ⁺ pro-T cells.

The functional role of the IL-2/IL-2R β interaction on pro-T cells indicated that IL-2 might be available in the thymus at an early developmental stage. Indeed, analysis of IL-2 gene expression revealed that the levels of IL-2 mRNA found in freshly isolated pro-T cells were similar to those found in mitogen-activated T lymphocytes, whereas IL-2 transcripts were undetectable in resting peripheral T lymphocytes (Fig. 3a).

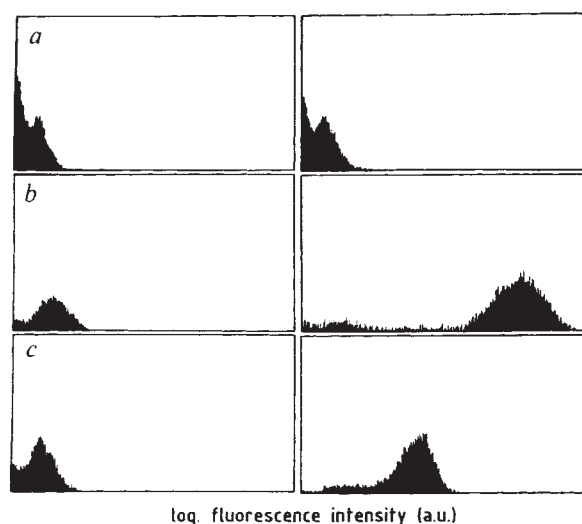


FIG. 1 IL-2 induced expression of IL-2R α in human pro-T cells. Pro-T cells, either freshly isolated, *a*, or cultured during 8 days with 1 nM human rIL-2, *b*, or with 1 nM rIL-2 and a 300-fold molar excess of anti-IL-2R α -monoclonal antibody, *c*, were analysed for the expression of IL-2R α (right panel). Staining with an irrelevant antibody gives the background fluorescence (left panel). METHODS. Normal human thymocytes were obtained from thymus fragments removed during corrective cardiac surgery of patients of aged one month to three years. Intrathymic stem cells, referred to as pro-T cells²², were isolated by consecutive treatments with anti-CD1, -CD4, -CD8, -CD3 and -CD2 monoclonal antibodies plus rabbit complement (Beringwerke), as previously described²². Recovered viable cells were treated again, in one step, with a cocktail of the indicated antibodies and incubated for 30 min at 4 °C with magnetic beads coated with affinity-purified sheep anti-mouse IgG (Dynabeads M-450; Dynal). This procedure allowed the isolation of a highly purified preparation of pro-T cells (>99% CD1⁺ 2⁺ 3⁺ 4⁺ 8⁺). Pro-T cell cultures were set up in 24-well microplates (Costar) (2×10^6 cells ml⁻¹) in RPMI-1640 medium (GIBCO) containing 10% FCS (GIBCO) and supplemented with either 1 nM human rIL-2 (Hoffman-La Roche) (cellular recovery ~500%) or with 1 nM rIL-2 plus a 300-fold molar excess of affinity-purified anti-IL-2R α (H-108)²⁶ monoclonal antibody (cellular recovery ~300%). Either freshly isolated or cultured pro-T cells were washed twice in RPMI-1640 medium, incubated for 3 min at 4 °C in 0.15 M NaCl, 0.1 M glycine (pH 4) (glycine buffer) and washed again three times to remove bound anti-IL-2R α antibody molecules. Cells were then sequentially incubated with 10 μ g ml⁻¹ of biotin-conjugated anti-IL-2R α (H-108) or irrelevant isotype-matching mouse monoclonal antibodies and phycoerythrin-conjugated streptavidin. Quantitation of the surface staining of 5×10^4 viable cells was performed in an EPICS Profile flow cytometer (Coulter Electronics). The figure shows the results of one representative experiment out of four.

Thus, IL-2 may be spontaneously produced by pro-T cells, allowing the acquisition of IL-2R α 'in vivo'.

To characterize the functional status of the IL-2-induced IL-2R α molecules, binding assays were performed with pro-T cells, after culture with 1 nM rIL-2. As compared with freshly isolated pro-T cells (Fig. 2a), cultured pro-T cells showed a 20-fold increase in the expression of low affinity (IL-2R α) receptors (21,200 sites per cell, K_d ~19 nM; Fig. 2d). More importantly, a marked induction of high-affinity binding sites was also detected (980 sites per cell, K_d ~12 pM; Fig. 2d). Binding of IL-2 to constitutive IL-2R β on pro-T cells leads to the activation of the IL-2R α gene and results in the assembly of the high-affinity IL-2R α / β heterodimer. These results were confirmed at the protein level by chemical crosslinking performed under high-affinity conditions (with 150 pM ¹²⁵I-labelled IL-2). Two major IL-2-associated bands of M_r ~90,000 (90 K) and 70K, corresponding to IL-2R β and IL-2R α , respectively, were detected in cultured pro-T cells (Fig. 3b), whereas no crosslinked species were detected when pro-T cells were incubated with a 500-fold molar excess of either anti-IL-2R α monoclonal antibody or unlabelled rIL-2 (Fig. 3b).

The proliferative potential of pro-T cells was investigated in culture by using a broad concentration range of rIL-2 (1-10⁵ pM), with or without saturating concentrations of anti-IL-

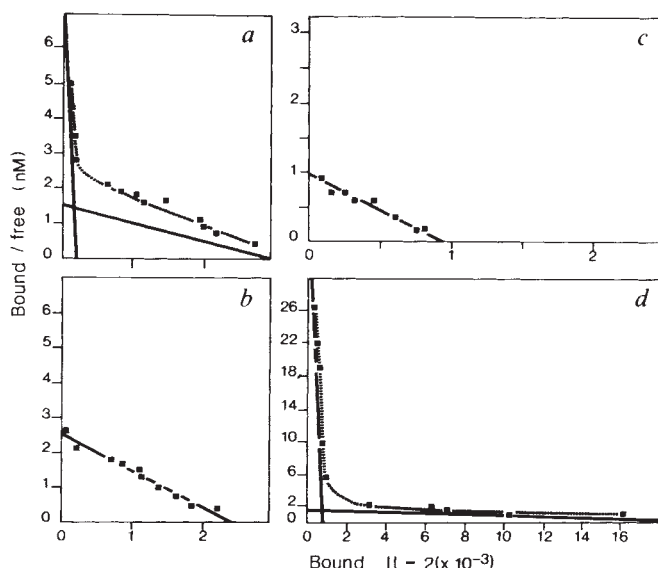


FIG. 2 Quantitative analysis of IL-2 binding sites displayed by freshly isolated pro-T cells, resting peripheral T cells and IL-2 cultured pro-T cells. IL-2 binding assays were performed in freshly isolated pro-T cells (*a*, *b*), in pro-T cells cultured with 1 nM rIL-2 for 8 days (*d*) and in resting peripheral T cells (*c*), either in the absence (*a*, *d*) or in the presence (*b*, *c*) of anti-IL-2R α .

METHODS. Pro-T cells were obtained as described in Fig. 1 legend, and highly purified resting peripheral T cells (96% CD2⁺, <0.1% Tac⁺, <0.5% CD16⁺) were isolated from peripheral blood by nylon wool columns followed by treatment with anti-CD16 and anti-IL-2R α antibodies and incubation with magnetic beads, as described in Fig. 1 legend. Aliquots of 2×10^7 cells were extensively washed and treated with glycine buffer as in Fig. 1 to remove bound IL-2 molecules. Cells (10^6) were then incubated with serial dilutions of ¹²⁵I-labelled human rIL-2 (1 pM–25 nM final concentration) (Amersham) for 1 h at 4 °C, in the absence or presence of a 500-fold molar excess of affinity-purified anti-IL-2R α (H-108) antibody, as previously described^{2,3}. Specifically bound and free ¹²⁵I-labelled IL-2 were determined by solid scintillation counting. The calculated values for the dissociation constant (K_d) and the number of binding sites per cell were derived by Scatchard computer analysis²⁷ of equilibrium binding data (solid lines). The dashed lines represent a computer-generated best fit to the experimental data. The data were corrected for nonspecific binding of IL-2, determined in the presence of a 250-fold molar excess of unlabelled rIL-2, and for the loss of ligand binding activity incurred during the IL-2 labelling procedures. The data are representative of three independent experiments.

2R α antibodies (3×10^{-7} M; Fig. 4). In the absence of anti-IL-2R α , IL-2 induced the proliferation of pro-T cells in a dose-dependent manner, maximal proliferation being achieved at doses of rIL-2 favouring intermediate-affinity binding (10 nM; Fig. 4a). Also, blocking of IL-2R α molecules did not affect cell proliferation at doses of rIL-2 high enough to saturate IL-2R β (<1 nM), whereas a significant inhibition was observed at low doses of rIL-2 (<500 pM), sufficient to bind to high-affinity IL-2R α/β heterodimers (Fig. 4a). By contrast, a fifteenfold lower concentration of rIL-2 was required to induce maximal proliferation of activated T cells expressing high-affinity IL-2R (100% Tac⁺) and, therefore, a shift in the IL-2 dose-response curve was induced by anti-IL-2R α . Ten- to fifteenfold higher concentrations of IL-2, sufficient to saturate the IL-2R β , were then required to induce T-cell growth (Fig. 4c). Similar inhibition patterns were observed for resting T lymphocytes (Fig. 4b), supporting the reported IL-2-dependent induction of high-affinity receptors on these cells¹⁴. The discrete proliferation observed in resting T cells when compared with pro-T cells (Fig. 4) may reflect their lower expression of IL-2R β (900 compared with 2,300 sites per cell).

Taken together, these results indicate that both intermediate- and high-affinity IL-2R can be involved in the IL-2-promoted proliferation of pro-T cells, indicating that, before acquisition of IL-2R α/β , IL-2R β alone can mediate signal transduction of IL-2. The observation that even without exogenous IL-2 supplementation, pro-T cells developed proliferative responses which can be blocked by anti-IL-2R α monoclonal antibody, however, argues that the spontaneous and 'physiological' growth of these cells may be mediated mainly by the high-affinity form of the IL-2R. Our data, therefore, provide evidence for the involvement of an IL-2-dependent autocrine pathway in the intrathymic

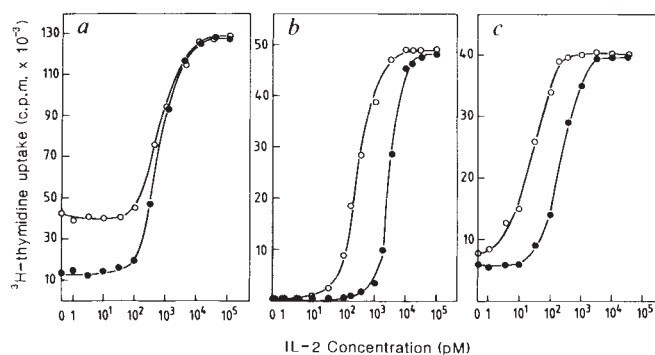


FIG. 4 IL-2 induced proliferation of pro-T cells. *a*, Pro-T cells; *b*, highly purified resting peripheral T cells; and *c*, phytohaemagglutinin-activated T cells were cultured in flat-bottomed 96-well microtitre plates (Costar) in RPMI-1640 medium at 10^6 cells ml^{-1} (*a, b*) or 10^5 cells ml^{-1} (*c*) with 3.0×10^{-7} M normal mouse affinity-purified IgG (○) or anti-IL-2R α (H-108) antibody (●), in the presence of different concentrations of rIL-2 ($1-10^5$ pM). Proliferative responses were analysed by [^3H]thymidine incorporation at day 5 of culture, after addition of 1 μCi per well of [^3H]thymidine (Amersham) for the last 18 h. Each value represents the mean of triplicate cultures. Standard deviations were $\leq 10\%$.

development of mature T cells. As previously proposed²³, such a nonspecific activation of polyclonal growth would operate selectively early in T-cell development, given the constitutive expression of mature IL-2 mRNA by pro-T cells. In agreement with this, high levels of IL-2 transcripts have been detected in 15-day fetal thymocytes, whereas expression decreases dramatically by day 16 up until birth²⁴.

Although the activation signals inducing IL-2 gene expression within the thymus are as yet unknown, recent results indicate that cellular interactions involving thymic stromal components and production of lymphokines other than IL-2, such as IL-1, play an essential role in this process²⁵. Thus, IL-1 appears to induce a marked activation of IL-2 gene in precursor thymocytes (E. Rothenberg, personal communication). In addition, we have observed that both IL-1 and IL-3 can regulate IL-2R α expression on pro-T cells (unpublished data). Although the possibility that this induction results from endogenous IL-2 production in the culture cannot be ruled out, it seems likely that more than one cytokine pathway may allow the acquisition of an IL-2-responsive state which, in turn, would ensure polyclonal expansion of T-cell precursors and T-cell differentiation. The autocrine behaviour that we propose occurs in T-cell differentiation could possibly be a common developmental strategy for other haemopoietic lineages. □

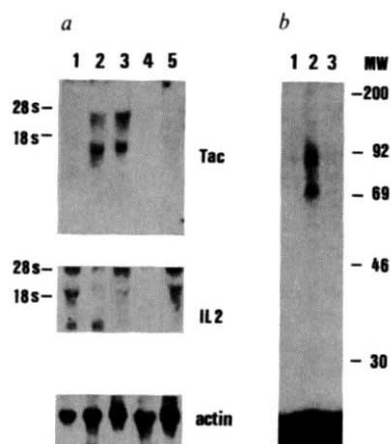


FIG. 3 Constitutive expression of IL-2 transcripts in T-cell precursors and induction of IL-2 gene expression. Acquisition of high-affinity IL-2R α/β induced by IL-2/IL-2R β interactions. *a*, Northern blot analysis of RNA obtained from freshly isolated pro-T cells (lane 1), phytohaemagglutinin-activated normal peripheral T cells (lane 2), pro-T cells cultured with 1 nM rIL-2 for 8 days (lane 3), JY B cells (lane 4) and resting peripheral T cells (lane 5). RNA (12 μg) was loaded on a formaldehyde-agarose gel, transferred to nylon membranes and hybridized with the ^{32}P -labelled (nick-translated) Tac-2A probe²⁸, the IL-2 probe²⁹, or an actin probe used as control, as previously described²². *b*, Chemical crosslinking of ^{125}I -labelled IL-2 to IL-2-treated pro-T cells. Pro-T cells, cultured for 8 days with 1 nM rIL-2 (10^7 cells) and washed with glycine buffer as described in Fig. 2 legend, were incubated with ^{125}I -labelled human rIL-2 (150 pM) in the absence (lane 2) or presence of a 500-fold molar excess of either unlabelled rIL-2 (lane 1) or anti-IL-2R α (H-108) monoclonal antibodies (lane 3). Disuccinimidyl suberate (Pierce) (0.3 nM) was used to covalently crosslink ^{125}I -labelled IL-2 to the surface of intact cells, as previously described⁹. Cells were solubilized in 1% SDS, 5% 2-mercaptoethanol sample buffer and analysed on 8% SDS-polyacrylamide gels. Similar results were obtained in four independent experiments.

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- Smith, K. A. *Immunol. Rev.* **51**, 335-357 (1980).
- Robb, R. J., Munck, A. & Smith, K. A. *J. exp. Med.* **154**, 1455-1474 (1981).
- Robb, R. J., Greene, W. C. & Rusk, C. M. *J. exp. Med.* **160**, 1126-1146 (1984).
- Leonard, W. J. *et al. Nature* **300**, 267-269 (1982).
- Sharon, M., Klausner, R. D., Cullen, B. R., Chizzonite, R. & Leonard, W. J. *Science* **234**, 859-863 (1986).
- Tsuda, M., Kozak, R. W., Goldman, C. K. & Waldmann, T. A. *Proc. natn. Acad. Sci. U.S.A.* **83**, 9694-9698 (1986).
- Teshigawara, K., Wang, H., Kato, K. & Smith, K. A. *J. exp. Med.* **165**, 223-238 (1986).
- Wang, H. M. & Smith, K. A. *J. exp. Med.* **166**, 1055-1069 (1987).
- Dukovich, M. *et al. Nature* **327**, 518-522 (1987).
- Robb, R. J., Rusk, C. M., Yodoi, J. & Greene, W. C. *Proc. natn. Acad. Sci. U.S.A.* **84**, 2002-2006 (1987).
- Robb, R. J. & Greene, W. C. *J. exp. Med.* **165**, 1201-1206 (1987).
- Smith, K. A. *Immun. Today* **9**, 36-37 (1988).
- Tanaka, T., Saki, O., Doi, S., Negoro, S. & Kishimoto, S. *J. Immun.* **140**, 470-473 (1988).
- Bich-Thuy, L. T. *et al. J. Immun.* **139**, 1550-1556 (1987).
- Meier, S. C. *et al. Proc. natn. Acad. Sci. U.S.A.* **81**, 1509-1513 (1984).
- Raulet, D. H. *Nature* **314**, 101-103 (1985).
- Ceredig, R., Lowenthal, J. W., Nabholz, M. & MacDonald, H. R. *Nature* **314**, 98-100 (1985).
- de la Hera, A. *et al. Eur. J. Immun.* **16**, 653-658 (1986).
- Shimorkevitz, R. P., Huscman, L. A., Bevan, M. J. & Crispe, I. N. *Nature* **329**, 157-159 (1987).
- Jenkinson, E. J., Kingston, R. & Owen, J. T. *Nature* **329**, 160-162 (1987).
- Toribio, M. L. *et al. Immunol. Rev.* **104**, 55-79 (1988).
- Toribio, M. L. *et al. J. exp. Med.* **168**, 2231-2249 (1988).

23. de la Hera, A., Toribio, M. L., Marcos, M. A. R., Márquez, C. & Martínez-A., C. *Eur. J. Immun.* **17**, 683-687 (1987).
 24. Carding, S. R. *et al. Proc. natn. Acad. Sci. U.S.A.* **86**, 3342-3345 (1989).
 25. de la Hera, A., Toribio, M. L. & Martínez-A., C. *International Immun.* (in the press).
 26. Carrera, A. C., Sánchez-Madrid, F., López-Botet, M., Bernabeu, C. & O. de Landázuri, M. *Eur. J. Immun.* **17**, 179-186 (1987).
 27. Munson, P. J. & Rodbard, D. *Analyt. Biochem.* **107**, 220-239 (1980).
 28. Nikaído, T. *et al. Nature* **331**, 631-635 (1984).
 29. Taniguchi, T. *et al. Nature* **302**, 305-310 (1983).

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A glycopospholipid anchor is required for Qa-2-mediated T cell activation

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A NUMBER of lymphocyte surface proteins are anchored in the cell membrane by glycoposphatidyl inositol (known as GPI) linkages instead of hydrophobic protein domains^{1,2}. Treatment of mouse T lymphocytes with antibodies specific for two such proteins, Thy-1 and Ly-6, are known to induce proliferation^{3,4}. We have found that antibodies specific for Qa-2, a GPI-anchored class I histocompatibility antigen⁵, can also activate mouse T cells. To determine whether the GPI-anchor is important for this pathway of cell activation, we produced transgenic mice expressing either normal GPI-anchored Qa-2, or Qa-2 molecules with a membrane-spanning protein domain derived from H-2. Our studies show that only lymphocytes from transgenic mice carrying GPI-anchored forms of Qa-2 can be activated *in vitro* by Qa-2-specific antibodies. We also show that transgenic mouse T cells expressing a GPI-anchored form of H-2D^b can be activated by anti-H-2D^b antibodies. These results strongly indicate that the GPI-anchor is critical for this pathway of T cell activation.

Activation of T cells is normally the result of binding of antigen receptors to antigenic peptides presented in association with major histocompatibility antigens. A number of other T-cell membrane proteins (for example, CD2, CD3 and CD28) can also induce activation in response to specific antibodies⁶⁻⁸. These antibodies may therefore mimic the binding of signalling molecules to their natural ligands. Molecules such as CD2 are transmembrane proteins that require a cytoplasmic domain to function in signalling⁹. Some GPI-anchored membrane proteins, such as Thy-1, have also been shown to act as signalling molecules, despite the fact that the fatty acid anchoring group appears to associate with only one face of the lipid bilayer^{2,10}. This indicates the existence of a second type of transmembrane signal delivery pathway involving GPI-anchors¹¹.

In mice, the class I, H-2 K, D and L major histocompatibility antigens are transmembrane glycoproteins that are thought to present processed endogenous antigenic fragments to receptors on T cells. Antibodies against H-2 are not normally mitogenic for T cells but antibodies specific for Qa-2, a GPI-anchored class I molecule⁵, are. Purified T cells from C57BL/10, Qa-2⁺, mice were stimulated to divide by three Qa-2 specific monoclonal antibodies. T cells from BALB/cJ mice, which express fewer Qa-2 molecules, could also be activated under these conditions. Anti-immunoglobulin antibody and phorbol myristate acetate (PMA), an activator of protein kinase C, were both required

for cell division to occur. Specificity for Qa-2 could be demonstrated by examining the proliferative responses of T cells from recombinant mouse strains B6.K1 and B6.K2, which differ only in the Q region¹² of the major histocompatibility complex. T cells from B6.K2 mice, which express Qa-2, could be activated by Qa-2 specific antibodies, but T cells from B6.K1 mice, which lack the antigen, could not (Table 1). Both types of T cells, however, respond equally well to Thy-1 specific antibodies.

To determine whether the GPI membrane anchor is required for the triggering of T cells by Qa-2 specific antibodies, we constructed hybrid class I genes by recombining segments of Q9^b or Q7^b, which both encode GPI-anchored Qa-2 molecules¹³⁻¹⁵, with corresponding regions of H-2D^b. The products of hybrid genes QD1 and QD3 are shown in Fig. 1. A GPI-anchored Qa-2 molecule is encoded by QD1, and QD3 encodes a variant form of Qa-2 which spans the cell membrane using a transmembrane segment derived from H-2D^b. Transgenic mice were derived by introducing these constructs into fertilized oocytes from CBA/Ca mice, which lack the genes for Qa-2. As shown in Fig. 2, spleen cells from both CBA/Ca.QD1 and CBA/Ca.QD3 transgenic mice expressed molecules reactive with anti-Qa-2 antibodies. QD1 molecules could be removed from cell membranes by treatment with phosphatidylinositol-specific phospholipase C (PtdIns-PLC), indicating the presence of a GPI anchor, but QD3 molecules were completely resistant to the enzyme.

To confirm that QD3 molecules have a transmembrane orientation, we treated microsomal membranes from biosynthetically labelled CBA/Ca.QD3 spleen cells with proteinase K. Cytoplasmic domains of transmembrane proteins exposed on the microsomal surface immediately after synthesis are removed by the enzyme¹⁶. Gel electrophoresis shows that QD3 molecules were reduced in relative molecular mass by ~3,000 following proteinase K treatment, consistent with removal of the H-2D^b-derived cytoplasmic domain¹⁶. By contrast, the size of QD1 was unchanged (data not shown). This confirms that QD3 spans the membrane. Both QD1 and QD3 were found to be associated with beta-2 microglobulin, as are Qa-2 and H-2D^b (data not shown).

The proliferative responses of T cells from CBA/Ca, CBA/Ca.QD1 and CBA/Ca.QD3 mice treated with Thy-1 or

TABLE 1 Proliferation of mouse T cells in response to Qa-2 specific antibodies

Cells	Antibody	Specificity	[³ H]thymidine incorporation (c.p.m.)
C57BL/10	None	—	600
	Qa-m2	Qa-2	48,000
	Qa-m7	Qa-2	67,000
	D3.262	Qa-2	14,000
	H142.45	H-2K ^b , D ^b	<600
	B22.249	H-2D ^b	<600
	K9.136	H-2K ^b	<600
BALB/c	None	—	3,400
	Qa-m2	Qa-2	56,000
	KT16	Thy-1	104,000
B6.K1	Qa-m2	Qa-2	300
	KT16	Thy-1	66,000
B6.K2	Qa-m2	Qa-2	23,900
	KT16	Thy-1	68,000

T lymphocytes were isolated from mouse spleens by nylon wool fractionation followed by absorption to anti-immunoglobulin coated dishes. Cells were then incubated with stimulatory antibodies at 1 μ g ml⁻¹ for 30 min at 25 °C, washed then cultured for 48 h in 96-well costar plates at 5 $\times$ 10⁵ cells per well in the presence of rabbit anti-mouse immunoglobulin F(ab')₂ fragments (5 μ g ml⁻¹) and PMA (50 ng ml⁻¹). Cell cultures were pulsed with [³H]thymidine (1 μ Ci, for 2 h), collected and counted.

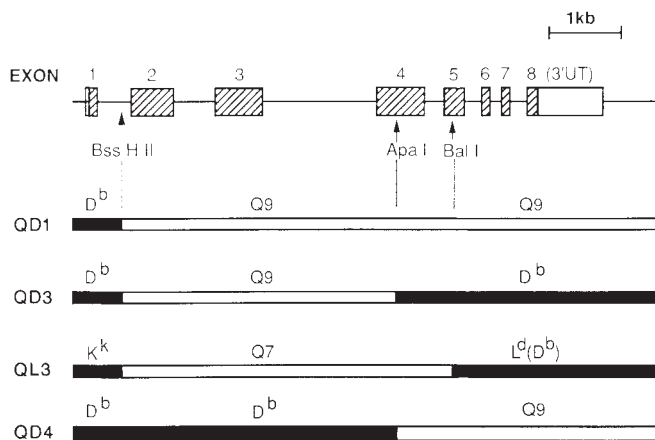


FIG. 1 Construction of *H-2/Qa-2* hybrid genes. Subclones of *H-2D^b*, *Q7^b* and *Q9^b* genes were recombined at the *Bss*HII, *Apa*I and *Bal*I sites indicated to construct the hybrid genes. Plasmid sequences were removed before injection into pronuclei of fertilized CBA/Ca mouse embryos using standard procedures. Transgenic *QD1*, *QD3*, and *QL3* mice were identified by hybridization of DNA from tail biopsy to a probe for the *Q9* large intron (between exons 3 and 4). *QD4* mice were identified using a 0.5 kb *Bam*HI fragment of *H-2D^b* from the 5' flanking region²⁰. UT, untranslated.

Qa-2 specific antibodies are shown in Table 2. Antibodies specific for *Qa-2* were mitogenic for T cells from CBA/Ca.QD1, but not CBA/Ca or CBA/Ca.QD3 mice. Proliferation of CBA/Ca.QD1 cells was abrogated by pretreatment with PtdIns-PLC. T cells from all three mouse lines responded well to Thy-1 specific antibodies, confirming that this activation pathway is not distorted by the presence of the transgene or its product. These results suggest that binding of anti-*Qa-2* antibodies is only mitogenic for T cells if the target antigen is anchored in the membrane with a GPI linkage.

In addition to their membrane anchors, QD1 and QD3 differ by eight amino acids in their membrane-proximal alpha-3 protein domains. To investigate whether T cells expressing membrane-spanning class I molecules with extracellular domains entirely of *Qa-2* origin would proliferate in response to anti-*Qa-2* antibodies, we produced transgenic mice expressing another construct, *QL3*. As shown in Fig. 1, the entire extracellular section of *QL3* is derived from *Qa-2*. The remainder of the molecule, including part of the membrane-spanning segment and the cytoplasmic domain, is from *H-2L^d*. *QL3* molecules were stably expressed as transmembrane glycoproteins on the surface of transgenic T cells (Fig. 2), despite the presence of an

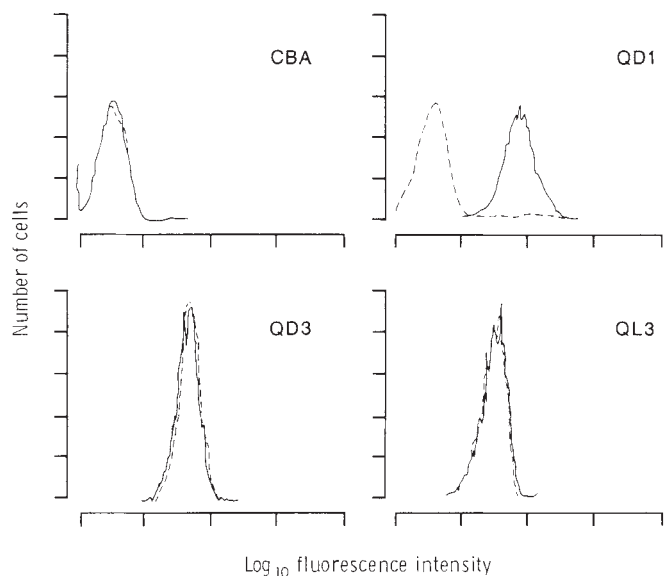


FIG. 2 Flow cytometry of hybrid class I molecules on transgenic T cells. T cells from transgenic mouse spleens were gated using biotinylated L3T4 and Ly-2 specific antibodies which were developed with streptavidin phycoerythrin. Transgene products on T cells were stained using *Qa-m2* antibody and rabbit antimouse immunoglobulin conjugated to fluorescein isothiocyanate. Solid lines show untreated cells, broken lines show cells after treatment with PtdIns-PLC before addition of antibody. Conjugate-only controls were indistinguishable from CBA/Ca profiles and therefore are not shown.

intramembrane aspartic acid residue, encoded by *Q7^b*, which was previously shown to be important for the addition of the GPI anchor¹⁷. T cells from CBA/Ca.QL3 and CBA/Ca.QD3 transgenic mice behaved similarly in the proliferation assay. Both gave a strong proliferative response to anti Thy-1 but no response to anti-*Qa-2* antibodies. As the entire extracellular portions of QD1 and QL3 differ by only a single amino acid at position 173 (alpha-2 domain) owing to a single base change between *Q7* and *Q9* (ref. 15), this strongly suggests that it is the GPI anchor that endows QD1 with the ability to deliver activation signals.

If GPI membrane anchors can confer transmembrane signalling capability on membrane proteins it should be possible to create new signal delivery structures by attaching phospholipid anchors to normally non-mitogenic molecules. To test this we produced one further transgenic mouse line, CBA/Ca.QD4, which expresses a GPI-anchored derivative of the *H-2D^b*

TABLE 2 Response of transgenic mouse T cells to class I-specific antibodies

Experiment 1	Antibody	Specificity		³ H]Thymidine incorporation (c.p.m.)		
				CBA/Ca	QD1	QD3
Experiment 1	KT16	Thy-1	-	260 (260)	340 (540)	300 (270)
	Qa-m2	Qa-2	-	35,400 (6,400)	73,000 (34,200)	95,400 (11,700)
				170 (70)	28,670 (147)	80 (90)
Experiment 2	Antibody	Specificity				
				C57BL/10	QL3	QD4
Experiment 2	KT16	Thy-1	-	ND	2,400	6,800
	Qa-m2	Qa-2	-	124,600 (40,800)	116,000 (152,000)	70,100 (34,000)
	141.30	H-2D ^b	-	29,800 (7,300)	2,200 (2,100)	5,100 (ND)
				5,100 (6,800)	5,700 (ND)	25,400 (10,300)

Conditions as for Table 1. In parentheses, cells were pretreated with 0.1U ml⁻¹ *Bacillus thuringiensis* PtdIns-PLC (1 h, 37 °C) and washed before adding stimulatory antibody.

molecule (Fig. 1). We found that T cells from these animals could be activated by antibodies against H-2D^b (Table 2). The antibody used, H141.30, is specific for the two outermost protein domains of H-2D^b (ref. 18). This was demonstrated by a lack of reactivity with cells carrying H-2L^d (ref. 18), which has an identical sequence to H-2D^b in the alpha-3 domain. Antibody H141.30 does not cross-react with Qa-2, as indicated by its lack of reactivity with CBA/Ca.QD1 spleen cells (data not shown) and did not cause proliferation of C57BL/10 T cells, which express conventional H-2D^b molecules (Table 2).

These activation data can best be explained by involvement of phospholipid membrane anchors in the process of transmembrane signalling and thus constitute direct evidence of a biological function associated with a GPI membrane anchor. Other

potential attributes of GPI linkages, such as increased lateral mobility of proteins in the plane of the membrane and their potential for selective release by phospholipases^{1,2}, have not yet been shown to have functional importance. By contrast, there is evidence suggesting the involvement of GPI anchors in cell activation¹¹. First, treatment of lymphocytes with PtdIns-PLC reduces their response to mitogens⁵. Second, T cell lines defective in anchor biosynthesis show reduced lymphokine production when stimulated with antigen¹⁹. The precise mechanism by which GPI anchors participate in cell triggering phenomena is still unclear, but their involvement in the activation of T lymphocytes suggests that they may be important for the regulation of cell proliferation during the immune response. □

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- Low, M. *Biochem. J.* **244**, 1-13 (1987).
- Ferguson, M. A. J. & Williams, A. F. *Rev. Biochem.* **57**, 285-320 (1988).
- Kroczyk, R. A., Gunther, K. C., Seligmann, B. & Shevach, E. M. *J. Immunol.* **136**, 4379-4384 (1986).
- Yeh, E. T. H., Reiser, H., Daley, J. & Rock, K. L. *J. Immunol.* **138**, 91-97 (1987).
- Stiernberg, J., Low, M. G., Flaherty, L. & Kincade, P. W. *J. Immunol.* **138**, 3877-3884 (1987).
- Ledbetter, J. D. *et al. Eur. J. Immunol.* **18**, 1601-1608 (1988).
- Van Wauwe, J. P., De May, J. R. & Goossens, J. G. *J. Immunol.* **124**, 2708-2713 (1980).
- Hara, T., Fu, S. M. & Hansen, J. A. *J. exp. Med.* **161**, 1513-1524 (1985).
- He, Q., Beyers, A. D., Barclay, A. N. & Williams, A. F. *Cell* **54**, 979-984 (1988).
- Gunther, K. C. *et al. Nature* **326**, 505-507 (1987).
- Robinson, P. J. & Spencer, S. C. *Immunol. Lett.* **19**, 85-94 (1988).
- Flaherty, L. *Immunogenetics* **3**, 533-539 (1976).

- Waneck, G. L., Sherman, D. H., Calvin, S., Allen, H. & Flavell, R. A. *J. exp. Med.* **165**, 1358-1370 (1987).
- Mellor, A. L., Antoniou, J. & Robinson, P. J. *Proc. natn. Acad. Sci. U.S.A.* **82**, 5920-5924 (1985).
- Devlin, J. J., Weiss, E. H., Paulson, M. & Flavell, R. A. *EMBO J.* **4**, 3203-3207 (1985).
- Dobberstein, B., Garoff, H., Warren, G. & Robinson, P. *Cell* **17**, 759-769 (1979).
- Waneck, G. L., Stein, M. E. & Flavell, R. A. *Science* **241**, 697-699 (1988).
- Lemke, H., Hämmerling, G. J. & Hämmerling, U. *Immunol. Rev.* **47**, 175-206 (1979).
- Yeh, E. T. H., Reiser, H., Bamezai, A. & Rock, K. L. *Cell* **62**, 665-674 (1988).
- Weiss, E. H., *et al. Nature* **310**, 650-655 (1984).

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Purified inositol 1,4,5-trisphosphate receptor mediates calcium flux in reconstituted lipid vesicles

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INOSITOL 1,4,5-trisphosphate (Ins(1,4,5)P₃), a second messenger molecule involved in actions of neurotransmitters, hormones and growth factors, releases calcium from vesicular non-mitochondrial intracellular stores¹. An Ins(1,4,5)P₃ binding protein, purified from brain membranes², has been shown to be phosphorylated by cyclic-AMP-dependent protein kinase³ and localized by immunohistochemical techniques to intracellular particles associated with the endoplasmic reticulum⁴. Although the specificity of the Ins(1,4,5)P₃ binding protein for inositol phosphates and the high affinity of the protein for Ins(1,4,5)P₃ indicate that it is a physiological Ins(1,4,5)P₃ receptor mediating calcium release, direct evidence for this has been difficult to obtain. Also, it is unclear whether a single protein mediates both the recognition of Ins(1,4,5)P₃ and calcium transport or whether these two functions involve two or more distinct proteins. In the present study we report reconstitution of the purified Ins(1,4,5)P₃ binding protein into lipid vesicles. We show that Ins(1,4,5)P₃ and other inositol phosphates stimulate calcium flux in the reconstituted vesicles with potencies and specificities that match the calcium releasing actions of Ins(1,4,5)P₃. These results indicate that the purified Ins(1,4,5)P₃ binding protein is a physiological receptor responsible for calcium release.

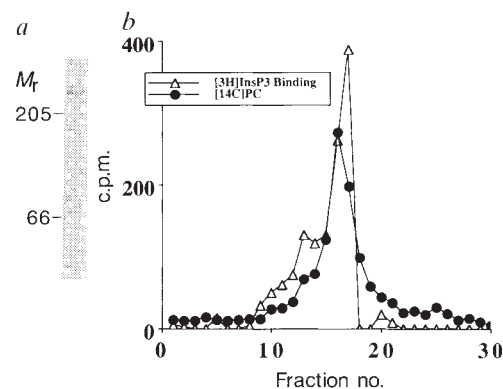
The Ins(1,4,5)P₃ receptor protein was solubilized using CHAPS as a detergent, rather than Triton X-100 which has been used previously², because Triton detergent is not compatible with the dialysis technique of vesicle reconstitution.^{5,6} The Ins(1,4,5)P₃ receptor protein was purified as previously described² with minor modifications (see Fig. 1). In the reconstitution experiments purified Ins(1,4,5)P₃ receptor protein was added to a mixture of CHAPS, solubilized phosphatidylcholine and phosphatidylserine, and the mixed micelles of protein and lipid were dialysed for 48 hr against a 1,000-fold excess of buffer. To determine whether the Ins(1,4,5)P₃ binding protein was incorporated into vesicles, we fractionated the vesicles on a continuous sucrose gradient and monitored [³H]Ins(1,4,5)P₃ binding as well as levels of [¹⁴C]phosphatidylcholine, which we used as a marker for the lipid vesicles (Fig. 1b). The [³H]Ins(1,4,5)P₃ binding activity and [¹⁴C]phosphatidylcholine co-migrated on the gradient and nearly 100% of the binding activity was recovered. Total protein concentration also co-migrated with the peak of [³H]Ins(1,4,5)P₃ binding activity (data not shown). Pure Ins(1,4,5)P₃ binding protein, dialysed in the absence of lipid, appeared to aggregate as it migrated to the densest portion of the gradient (data not shown). Lipid vesicles without receptor protein appeared slightly less dense than the protein containing vesicles (data not shown). Analysis of protein within the vesicles by SDS-PAGE revealed a single band with a relative molecular mass of 260,000 (260K) (Fig. 1a), the same as purified Ins(1,4,5)P₃ receptor protein not incorporated into vesicles². Thus, the reconstitution procedure results in the incorporation of Ins(1,4,5)P₃ binding protein into the vesicles with no change in molecular weight, and the protein retains its ability to bind Ins(1,4,5)P₃.

The properties of [³H]Ins(1,4,5)P₃ binding in the reconstituted vesicles were essentially the same as those of the purified unconstituted protein². Unlabelled Ins(1,4,5)P₃ inhibited [³H]Ins(1,4,5)P₃ binding 50% at ~40nM (Fig. 2). Of a variety of inositol phosphates examined, Ins(1,4,5)P₃ was the most potent inhibitor of binding with Ins(2,4,5)P₃ being the next potent. Both Ins(1,3,4)P₃ and Ins(1,3,4,5)P₄ were much less potent inhibitors of binding, whereas other inositol phosphates examined were essentially inactive (Table 1). Heparin, a known inhibitor of Ins(1,4,5)P₃ binding⁷ and Ins(1,4,5)P₃-mediated

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FIG. 1 Reconstitution of purified Ins(1,4,5) P_3 receptor protein. *a*, SDS-PAGE analysis of protein in reconstituted lipid vesicles (7.5% gel, Coomassie Brilliant Blue stain). *b*, Disposition of specific [3H]Ins(1,4,5) P_3 (open triangles) binding in continuous sucrose gradient (5–20%) fractionation of reconstituted proteoliposomes. Fraction number 1 corresponds to 20% sucrose or the bottom of the gradient, whereas fraction number 30 is 5% sucrose at the top of the gradient. The [^{14}C]Phosphatidylcholine (PC, closed circles) was used as a tracer for the lipid vesicles. Nonspecific [3H]Ins(1,4,5) P_3 binding was measured in the presence of 1 μM Ins(1,4,5) P_3 . This experiment was performed three times with the same results. Binding was determined in duplicate or triplicate with replicates varying <10%.

METHODS. Ins(1,4,5) P_3 receptor was purified as described² with the following modifications: CHAPS (1%; Sigma) was used as the detergent to allow dialysis for reconstitution, and the receptor was purified using only two affinity column chromatography steps². Partially purified receptor was obtained by fractionating CHAPS solubilized proteins on Heparin agarose (Sigma, Type II) as described². The purified receptor was obtained by fractionating the proteins which elute from heparin agarose on concanavalin sepharose². The reconstitution strategy was based on a previously described procedure used for the nicotinic acetylcholine receptor⁶. Solubilized liposomes were prepared by sonicating (for 12–20 min in a cylindrical bath sonicator) a mixture of phosphatidylcholine (in the presence or absence of tracer amounts of [^{14}C] phosphatidylcholine) and phosphatidylserine (Avanti Polar Lipids) at a ratio of 3:1, and a lipid concentration of 40 mg ml⁻¹ and in a buffer (buffer A) containing 50 mM NaCl, 50 mM KCl and 20 mM Tris-HCl (pH 7.4, 25 °C). Following sonication the lipid concentration was reduced to 20 mg ml⁻¹ by adding an equal volume of buffer A and CHAPS was added to bring the final detergent concentrations to 1%. This lipid mixture was then mixed with purified Ins(1,4,5) P_3 receptor (100 μg ml⁻¹ protein) or



partially purified (400 μg ml⁻¹) at a ratio of 1:1. After incubation on ice for 20 min, the mixture was dialysed (molecular mass cutoff, 10,000) against a 1,000-fold excess of buffer A supplemented with 1 mM EDTA, 1 mM EGTA and 2 mM 2-mercaptoethanol. The buffer was changed every 8 h for 48 h to effect detergent removal and vesicle formation. These vesicles were then loaded onto a 5–20% continuous sucrose gradient (30 ml) and centrifuged overnight (SW 28, 20,000 r.p.m. (140,000g) Beckman Ultracentrifuge). Fractions (1 ml) were collected; [^{14}C]phosphatidylcholine content and [3H]Ins(1,4,5) P_3 binding were measured. Protein concentration was determined by the method of Bradford²², and SDS-PAGE was by the method of Laemmli²³.

calcium release^{8–13}, completely inhibited binding at 100 μg ml⁻¹ (Table 1). As previously reported for the purified, unreconstituted Ins(1,4,5) P_3 binding protein², calcium (1 mM) had no effect on [3H]Ins(1,4,5) P_3 binding—which was markedly influenced by pH, more than doubling as pH was increased from 7.4 to 8.8 (data not shown).

To monitor calcium flux, reconstituted vesicles were incubated with $^{45}Ca^{2+}$ in the presence or absence of various concentrations of Ins(1,4,5) P_3 or other inositol phosphates (Figs. 2, 3; Table 1). At 10 μM Ins(1,4,5) P_3 the rate of $^{45}Ca^{2+}$ flux was stimulated at least fivefold, being half maximal within 4 s and complete within 10 s (Fig. 3). The very rapid kinetics of Ins(1,4,5) P_3 -stimulated $^{45}Ca^{2+}$ influx would be expected for ion channels reconstituted in small unilamellar liposomes⁶. Half maximal and maximal Ins(1,4,5) P_3 -stimulated $^{45}Ca^{2+}$ influx required ~40 nM and 1 μM Ins(1,4,5) P_3 , respectively, very similar to corresponding potencies for inhibiting [3H]Ins(1,4,5) P_3 binding (Fig. 2). The relative effects of other inositol phosphates on $^{45}Ca^{2+}$ flux also resembled their influences on [3H]Ins(1,4,5) P_3 binding, with Ins(1,4,5) P_3 being most potent and Ins(2,4,5) P_3 the only other inositol phosphate with activity (Table 1). These effects resemble the relative calcium-releasing potencies of inositol phosphates in several systems^{14–16}. Also, the relative potencies of the inositol

phosphates in mediating $^{45}Ca^{2+}$ release from cerebellar microsomes were the same as in the reconstituted vesicles (data not shown). Interestingly, whereas Ins(1,3,4) P_3 and Ins(1,3,4,5) P_4 , at concentrations of 10 and 20 μM respectively, produced significant inhibition of [3H]Ins(1,4,5) P_3 binding, they did not elicit a calcium flux (Table 1). Whether these results indicate that at those concentrations these substances are Ins(1,4,5) P_3 receptor antagonists is not clear. Heparin (100 μg ml⁻¹) completely blocked the ability of Ins(1,4,5) P_3 (1 μM) to stimulate a $^{45}Ca^{2+}$ flux (Table 1) with no effect on $^{45}Ca^{2+}$ flux in the absence of Ins(1,4,5) P_3 .

Although in these experiments we examined the effects of inositol phosphates on the influx of $^{45}Ca^{2+}$ into reconstituted vesicles, in other experiments we have shown similar effects on $^{45}Ca^{2+}$ release from reconstituted vesicles preloaded with $^{45}Ca^{2+}$. Ins(1,4,5) P_3 doses of 10 μM or 1 μM released, with rapid kinetics, the $^{45}Ca^{2+}$ from the inside of vesicles preloaded by freeze thaw (see Fig. 3 legend). When the partially purified preparations of the receptor (see Fig. 1 legend) were reconstituted in parallel with the purified receptor, the specific activity of [3H]Ins(1,4,5) P_3 binding increased 9.3-fold after the second affinity chromatography step, whereas the specific activity of Ins(1,4,5) P_3 -mediated calcium flux increased 10.3-fold. In

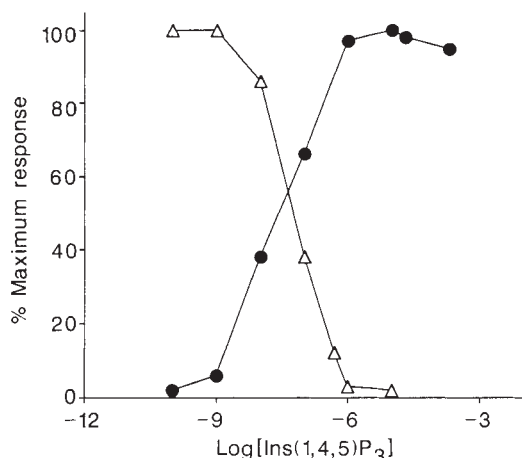


FIG. 2 Concentration-response relationships for Ins(1,4,5) P_3 inhibition of [3H]Ins(1,4,5) P_3 binding (Δ) and stimulation of $^{45}Ca^{2+}$ influx into vesicles ($\bullet$) reconstituted from purified Ins(1,4,5) P_3 receptor protein. Calcium flux was measured as described in the legend to Fig. 3, and the incubation time for calcium flux was 10 s. This experiment has been performed four times with essentially the same results. Measurements were made in triplicate or quadruplicate with replicates varying <10%.

experiments using lipid vesicles without incorporated Ins(1,4,5)P₃ receptor protein, no effects of inositol phosphates on the ⁴⁵Ca²⁺ flux were detected. Also, reconstitution of protein from column fractions containing no binding activity resulted in vesicles that had no Ins(1,4,5)P₃-stimulated ⁴⁵Ca²⁺ transport. To demonstrate that the intact vesicle structure is required for Ins(1,4,5)P₃ effects on calcium flux, we solubilized reconstituted vesicles with CHAPS (1%) and were no longer able to detect Ins(1,4,5)P₃-stimulated ⁴⁵Ca²⁺ flux. In addition we examined whether the ⁴⁵Ca²⁺ accumulated in the reconstituted vesicles after Ins(1,4,5)P₃ stimulation could be released by ionophores. We therefore collected the vesicles after passing them over the Dowex column, incubated them in the presence and absence of the calcium ionophore A23187 and then passed them over a second Dowex column. All of the ⁴⁵Ca²⁺ accumulated after Ins(1,4,5)P₃ stimulation was released by the ionophore.

Our ability to reconstitute Ins(1,4,5)P₃-stimulated calcium flux into lipid vesicles containing purified Ins(1,4,5)P₃ binding protein establishes that this protein is a physiological receptor mediating Ins(1,4,5)P₃ effects on calcium release. The close

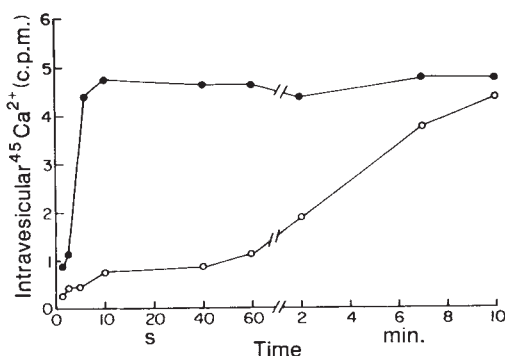


FIG. 3 Time course of ⁴⁵Ca²⁺ flux in vesicles reconstituted with purified Ins(1,4,5)P₃ receptor. Open symbols, accumulation of ⁴⁵Ca²⁺ in reconstituted vesicles in the absence of any agonist; closed symbols, ⁴⁵Ca²⁺ accumulated in the presence of 10 μM Ins(1,4,5)P₃. Data are expressed as c.p.m. (×1,000). This experiment has been replicated three times with similar results. Measurements were made in duplicate or triplicate with replicates varying <10%.

METHODS. Following reconstitution of the purified Ins(1,4,5)P₃ receptor as described in the legend to Fig. 1, vesicles were dialysed overnight in buffer A supplemented with 2 mM 2-mercaptoethanol to remove the calcium chelators which interfere with calcium flux measurements and to bring the final calcium concentration to nominally free levels of ~10 μM free calcium as measured by a calcium sensitive macroelectrode. Calcium flux was determined using tracer amounts of ⁴⁵Ca²⁺ and cation exchange chromatography. Calcium influx was measured by incubating 85 μl of reconstituted vesicles with 2 μCi ⁴⁵Ca²⁺ in the presence or absence of 10 μM Ins(1,4,5)P₃. The final reaction volume was 100 μl. After incubation for the indicated times, the flux reaction was stopped with a threefold excess of buffer A supplemented with 0.3 mM CaCl₂, 5 mM MgSO₄, 1 mM CdCl₂ and 100 μg ml⁻¹ heparin (Sigma) (Stop buffer). This 400 μl mixture of vesicles and stop buffer was immediately loaded onto a 3 ml column of Tris-Dowex (Sigma, Dowex 50-W X-8, pH 8.0) which had been equilibrated with buffer containing 200 mM sucrose, 20 mM Tris (pH 7.4, 25 °C) and 0.3% BSA. The column was immediately washed with 3.5 ml of the same buffer (without BSA) to wash the vesicles and the intravesicular ⁴⁵Ca²⁺ through the column. The ⁴⁵Ca²⁺ content of the vesicles was determined by liquid scintillation spectrometry. In some experiments, however, following the removal of extravesicular calcium by the Dowex column, the vesicles were immediately incubated in 3.5 ml of the sucrose containing buffer in the presence or absence of 10 μM A23187, a calcium selective ionophore. After a 10 min incubation at 22 °C, the vesicles were reloaded onto (and washed through) a second Dowex column (6 ml column volume) to determine the selective releasability of the accumulated calcium. For efflux measurements vesicles were loaded with ⁴⁵Ca²⁺ by rapid freeze thaw. After freeze thaw, vesicles were diluted fivefold into buffer A in the presence or absence of 10 μM Ins(1,4,5)P₃. After a 10 s incubation at 22 °C the flux reaction was stopped and the intravesicular ⁴⁵Ca²⁺ content was determined as described above.

TABLE 1 [³H]Ins(1,4,5)P₃ binding and Ins(1,4,5)P₃-stimulated ⁴⁵Ca²⁺ flux

Compound	Concentration (μM)	% Inhibition of [³ H]Ins(1,4,5)P ₃ binding	% Stimulation of ⁴⁵ Ca ²⁺ flux
Ins(1,4,5)P ₃	1	100	97
Ins(1,3,4)P ₃	10	40	0
Ins(2,4,5)P ₃	2	72	52
Ins(2)P	5	3	0
Ins(4)P	5	0	0
Ins(1,4)P ₂	10	5	0
Ins(1,3,4,5)P ₄	20	62	0
InsP ₅	10	12	0
InsP ₆	10	15	0
Heparin	100 μg ml ⁻¹	100	0
Ins(1,4,5)P ₃ + heparin	100 μg ml ⁻¹	—	3

Calcium flux and [³H]Ins(1,4,5)P₃ binding were determined as described in the legends to Figs 1 and 2. Flux measurements were made after a 10 s incubation as in Fig. 3. These experiments have been performed three times in triplicate or quadruplicate with replicates varying by <15%.

correlation between potency of inositol phosphates to influence ligand binding and calcium flux supports this conclusion. Our findings also establish that a single protein mediates both recognition of Ins(1,4,5)P₃ and the stimulation of calcium flux. In this way the Ins(1,4,5)P₃ receptor resembles neurotransmitter-gated ion channels, such as the nicotinic acetylcholine⁶, GABA (γ-aminobutyric acid)-benzodiazepine¹⁷, and glycine¹⁷ receptors, in which a single protein-complex contains recognition sites both for the neurotransmitter and associated ion channels. These neurotransmitter receptors are comprised of several distinct subunits, whereas the Ins(1,4,5)P₃ receptor seems to be a tetramer of four identical subunits². The neurotransmitter receptors are integral proteins of the plasma membrane with recognition sites on the extracellular surface, whereas the Ins(1,4,5)P₃ receptor is a novel intracellular receptor with the recognition site for Ins(1,4,5)P₃ on the cytoplasmic side. In this way the Ins(1,4,5)P₃ receptor resembles the recently characterized¹⁸⁻²⁰ and cloned²¹ ryanodine receptor, which mediates calcium flux from the sarcoplasmic reticulum of skeletal muscle. The Ins(1,4,5)P₃ receptor also resembles the ryanodine receptor in having a high relative molecular mass, and a similar proposed subunit structure and chromatographic behaviour¹⁸⁻²¹. It is likely, therefore, that these two receptors are members of a new class of intracellular ligand-gated ion channels. □

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- Berridge, M. J. *Rev. Biochem.* **56**, 159-193 (1987).
- Supattapone, S., Worley, P. F., Baraban, J. M. & Snyder, S. H. *J. Biol. Chem.* **263**, 1530-1534 (1988).
- Supattapone, S. et al. *Proc. natn. Acad. Sci. U.S.A.* **85**, 8747-8750 (1988).
- Ross, C. A. et al. *Nature* **339**, 468-470 (1989).
- Jones, O. T., Earnest, J. P. & McNamee, M. G. in *Biological Membranes: A Practical Approach* (eds Findlay, J. B. C. & Evans, W. H. (IPL, Oxford, 1987).
- Huganir, R. & Racker, E. *J. Biol. Chem.* **257**, 9372-9378 (1982).
- Worley, P. F. et al. *J. Biol. Chem.* **262**, 12132-12136 (1987).
- Hill, T., Berggren, P. O. & Boynton, A. *Biochem. biophys. Res. Commun.* **149**, 897-901 (1987).
- Cullen, P. J., Comerford, J. G. & Dawson, A. P. *FEBS Lett.* **228**, 57-59 (1988).
- Nilsson, T., Zwiler, J., Boynton, A. L. & Berggren, P. O. *FEBS Lett.* **229**, 211-214 (1988).
- Joseph, S. K. & Rice, H. L. *Molec. Pharm.* **35**, 355-359 (1989).
- Ghosh, T. K. et al. *J. Biol. Chem.* **263**, 11075-11079 (1988).
- Guillemette, G., Lamontagne, S., Boulay, G. & Mouillac, B. *Molec. Pharm.* **35**, 339-344 (1989).
- Ghosh, T. K., Mullaney, M. M., Tarazi, F. I. & Gill, D. L. *Nature* **340**, 236-239 (1989).
- Irvine, R. F. *Biochem. Soc. Trans.* **17**, 6-9 (1989).
- Nahorski, S. R. *Trends pharm. Sci.* **10**, 139-144 (1989).
- Barnard, E. A., Darlison, M. G. & Seeburg, P. *Trends Neurosci.* **10**, 502-509 (1987).
- Inui, M., Saito, A. & Fleischer, S. *J. Biol. Chem.* **262**, 1740-1747 (1987).
- Lai, G. A., Erickson, H. P., Roussear, D., Liu, Q.-Y. & Meissner, G. *Nature* **331**, 315-319 (1988).
- Imagawa, T., Smith, J. S., Coronado, R. & Campbell, K. P. *J. Biol. Chem.* **262**, 16636-16643 (1987).
- Takehisa, H. et al. *Nature* **339**, 439-445 (1989).
- Bradford, M. M. *Analyt. Biochem.* **72**, 248-254 (1976).
- Laemmli, U. K. *Nature* **227**, 680-685 (1970).

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Nature and site of phospholamban regulation of the Ca^{2+} pump of sarcoplasmic reticulum

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THE rapid removal of Ca^{2+} ions from the cytosol, necessary for the efficient relaxation of cardiac muscle cells, is performed by the Ca^{2+} -pumping ATPase of the sarcoplasmic reticulum. The calcium pump is activated by cyclic AMP- and calmodulin-dependent phosphorylation of phospholamban, an integral membrane protein of the sarcoplasmic reticulum. Using a heterobifunctional crosslinking agent which can be cleaved and photoactivated, we provide evidence for a direct interaction between the two proteins. Only the non-phosphorylated form of phospholamban interacts with the ATPase, demonstrating that phospholamban is an endogenous inhibitor that is removed from the ATPase by phosphorylation. Non-phosphorylated phospholamban interacts only with the calcium-free conformation of the ATPase and is released when it is converted to the calcium-bound state. We localized the site of interaction to a single peptide isolated after cyanogen bromide cleavage of the ATPase. The peptide derives from a domain just C-terminal to the aspartyl phosphate of the active site. This domain is unique to ATPases of the sarcoplasmic reticulum in that it has no homology with any other phosphorylation-type ion pump. The domain occurs in both slow- and fast-twitch isoforms of the ATPase, even though phospholamban is not expressed in fast-twitch muscles.

Phospholamban (PLN) is a complex of five identical amphipathic peptides of relative molecular mass (M_r) ~6,000 (ref. 1). In its hydrophilic domain each monomer contains adjacent serine and threonine residues that can be selectively phosphorylated by the cAMP-dependent and calmodulin-dependent kinases respectively². Phosphorylation of PLN is important in the mechanical response of cardiac muscle to stimulation by beta-agonists^{3,4}. We isolated phospholamban oligomer⁵ and conjugated the ϵ -amino group of its single lysine residue, situated near the N-terminus in the hydrophilic domain, to the *N*-oxysuccinimide moiety of the heterobifunctional Denny-Jaffe crosslinking reagent⁶ to form a stable amide linkage. When conjugated with the Denny-Jaffe reagent, PLN remains oligomeric. The reagent contains a cleavable distal portion carrying a photoactivatable aryl azide and a radioactive ^{125}I label. It has been postulated that PLN, which is present in roughly stoichiometric amounts with the calcium pump (that is, one monomer per pump unit), interacts directly with the enzyme to induce a conformational change in it. Light activation of conjugated PLN incubated with purified cardiac sarcoplasmic reticulum (SR) ATPase resulted in the formation of a complex (Fig. 1, lane 3). But when PLN was pre-phosphorylated by the cAMP-dependent kinase, or when Ca^{2+} ions were added to the incubation mixture, there was no crosslinking with the ATPase (Fig. 1; lanes 1, 2 and 4). Complex formation therefore occurs only when PLN is in the unphosphorylated state. Experiments using reconstitution techniques⁷, monoclonal antibodies against PLN⁸ or controlled tryptic digestion of PLN⁹ have indicated that PLN is an inhibitor of the calcium pump and that phosphorylation removes this inhibition, a suggestion that is now strongly supported by our result.

Addition of 100 μM Ca^{2+} completely inhibits complex formation between the two proteins (Fig. 1). When Ca^{2+} ions bind to

high-affinity sites¹⁰, the ATPase undergoes a large conformational change to assume the E_1 conformation, which can then be phosphorylated by ATP during the calcium transport cycle¹¹. We investigated whether inhibition of the PLN interaction by calcium resulted from conversion of the ATPase into the E_1 state by studying the calcium dependence of the hydrolytic activity of the pump in parallel with labelling experiments. The hydrolytic activity can be taken as an indicator of the saturation level of the high-affinity Ca^{2+} -binding sites¹¹. Figure 2 shows that the activation of the pump by Ca^{2+} is inversely correlated with its ability to interact with PLN, so only the E_2 (Ca^{2+} -free) conformation of the ATPase can bind PLN. Interaction with non-phosphorylated PLN therefore shifts the equilibrium of the pump in favour of the E_2 form, slowing down the $\text{E}_2 \rightarrow \text{E}_1$ conversion and raising the apparent K_m for calcium of the enzyme. This would explain earlier observations on the binding kinetics of Ca^{2+} to the cardiac ATPase: PLN phosphorylation, which results in the removal of PLN from its binding site on the pump, was shown to increase the rate of binding of Ca^{2+} ions¹² and the apparent affinity for calcium of the ATPase^{13,14}. Changes in the electrostatic properties of the SR membrane during PLN phosphorylation also contribute to the increased calcium affinity of the ATPase¹⁵.

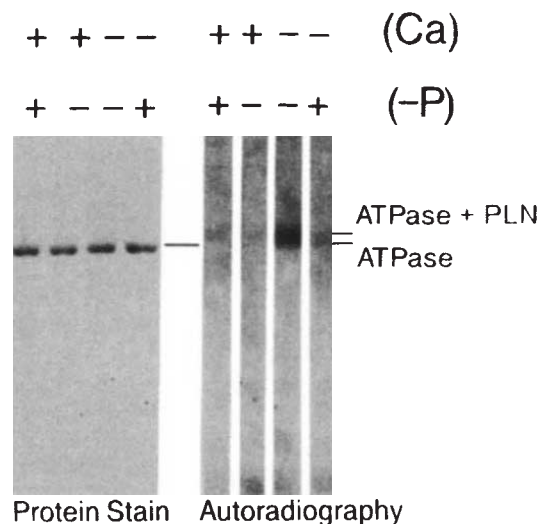


FIG. 1 Crosslinking of Denny-Jaffe labelled PLN to the isolated SR ATPase. *a*, Protein-stained SDS-polyacrylamide gel. *b*, Autoradiograph of same gel. Conjugated PLN crosslinked to the SR Ca^{2+} -ATPase (lane 3). The interaction between the two proteins was strongly inhibited by pre-phosphorylation of PLN (lanes 1 and 4) or by Ca^{2+} ions (lanes 1 and 2).

METHODS. PLN was purified¹ and labelled with the Denny-Jaffe reagent in 0.1% C_{12}E_8 detergent (dodecyl octaoxyethylene glycol monoether), 10 mM phosphate buffer, pH 6.8, in the dark¹⁷. Labelling was carried out in the presence of cyclohexadione to block all arginine residues. As the N-terminus is blocked, the only group left to react with the reagent is the single lysine of PLN. Using cold Denny-Jaffe reagent, about 5 mol azide per PLN pentamer could be incorporated. SR ATPase was isolated from rabbit heart according to ref. 18. The ATPase preparation (~1 mg ml^{-1}) was crosslinked¹⁷ to the conjugated PLN (stoichiometric ratio 1:1, pump to pentamer) in a medium composed of 20 mM Tris-maleate, pH 7.0, 0.1% C_{12}E_8 , 2.5 mM MgCl_2 , and in the presence of either 0.5 mM EGTA (lanes 3 and 4) or 0.1 mM Ca^{2+} (lanes 1 and 2). If necessary, PLN was pre-phosphorylated (lanes 1 and 4) using the catalytic subunit of the cAMP-dependent protein kinase. Results were identical when phosphorylation was carried out before or after conjugation of PLN to the Denny-Jaffe reagent. Samples of protein (10 μg) were then run on 10% polyacrylamide/SDS-gels¹⁹, stained with Coomassie brilliant blue (*a*), dried and autoradiographed (*b*). The molecular weight of the crosslinked ATPase population was altered by ~6,000 because boiling in sample buffer dissociates the PLN pentamer. The sample buffer contained dithiothreitol, which on heating partially reduces the azo crosslink, thus explaining the width of the radioactive band. The coupling efficiency was low (5–10%), which is usual in most photo-crosslinking reactions.

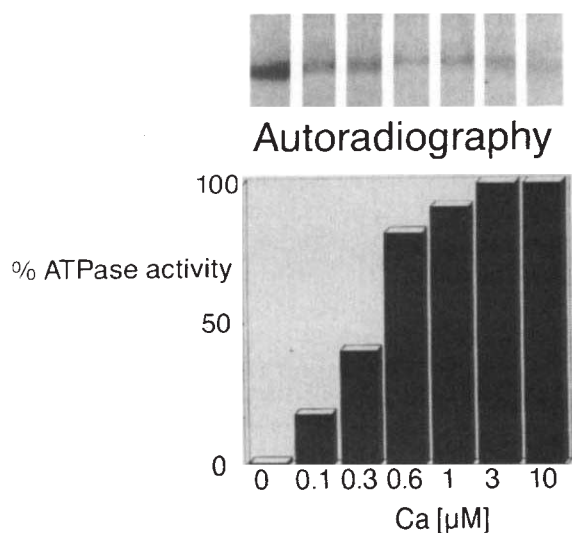


FIG. 3 Isolation and amino-acid sequence analysis of the PLN-binding peptide. **a**, Reverse-phase HPLC of peptides formed by cyanogen bromide digestion of ^{125}I -labelled SR ATPase. **b**, Re-chromatography on an ion-exchange column of the fractions from **a** that contained the radioactivity. Solid lines indicate absorbance at 206 nm and broken lines, radioactivity in c.p.m.

METHODS. Isolated cardiac SR Ca^{2+} -ATPase (10 mg) was photolysed in the presence of 200 μg non-phosphorylated, Denny-Jaffe-conjugated PLN, as described in the legend to Fig. 1. After reduction and carboxymethylation of thiol groups, PLN was cleaved off using 100 mM sodium dithionite¹⁷ and removed from the solution by dialysis (cut-off range 50,000 M_r). This cleavage leaves the ^{125}I label attached to the ATPase domain that interacts with PLN. The ATPase was then digested with CNBr and fractionated on a 300-Å, 7- μ , 250 $\times$ 10 mm C-18 RP column (0.1% trifluoroacetic acid in water to 100% *n*-propanol in 50 min). The broad radioactive peak was collected and diluted into 20% *n*-propanol, 10 mM Tris-HCl, pH 8.5 (buffer A), and then injected onto a Mono-Q column equilibrated in the same buffer. A gradient of 0–0.5 M NaCl in buffer A was run over 60 min. Radioactive fractions were desalted by reverse-phase HPLC and sequenced in an Applied Biosystems 470A sequencer with on-line detection. Half of the phenylthiohydantoin amino acids released per cycle were injected onto the 120A HPLC unit of the system for analysis, and the other half counted to determine radioactivity in a Beckman gamma counter.

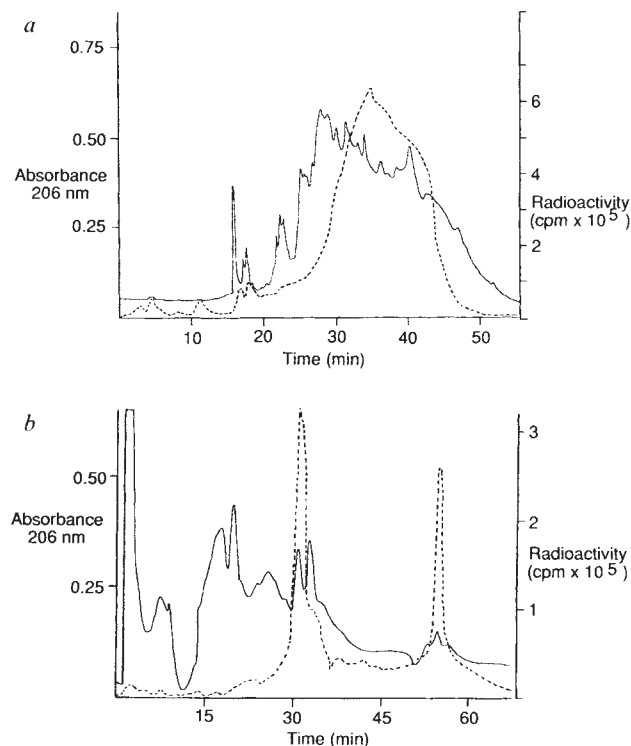
FLB-binding peptides:		slow twitch (rabbit)		FILDKVDGETCSLNEFTITGTYAPIGEVHKDDKPVKCHO			
		fast twitch (dog)		FTIDKVDGNLCVLNEFAITGTYAPEGEVLKNDKPIRS			
Ca (SR)	1	LGCTSVICSDKTGTLTNNQ	MSVCRMFI	FILDKVDGETCSLNEFTITGTYAPIGEVHKDDKPVKCHQTDGLVELATICALCNDSDALDNE	AKGVYKVGGEATE		
	2	LGCTSVICSDKTGTLTNNQ	MSVCKMFI	DKVDGDFCSLNEFTITGTYAPEGEVLKNDKPIRSQFGLVELATICALCNDSSLDNE	TKGVYKVGGEATE		
Ca (PM)	3	MGNATAICSDKTGTLTMNR	MTVVQAYINEK	HY	KKVPEPEAI	PPNLSYL	VTGISVNCA YTSKILPPEK EGGLPRHVGNKTE
Na/K	4	LGSTSTICSDKTGTLTNNR	MTVAHMFNDQIHEADTTENQ			SGVSFDKTSATWLALSRIAIGLCNRAVFAQQNDNLPILKRAVAGDASE	
	5	LGSTSTICSDKTGTLTNNR	MTVAHMFNDQIHEADTTENQ			SGISFDKTSLSWNLASRIAALCNRAVFAQQDQSVPLKISVAGDASE	
H	6	MSGVNMILCSDKTGTITLTK	MEIQEQCFTEEG			NDLKSTLVLAALAAKWRPPRDALDTMVLGAADLDE	
	7	LAGEVILCSDKTGTITLTK	LSLHEIYTVEGVS			PDDLMLTAPLAASRRKKGLDAIDKAFKLSLKQYPKAK	
K	8	AGDQVLLLDKTGTITLGN	RQASEFIPAQGV			EKTLADAQLASLADETPGRSIVILAKQRFNLRER	
	9	ANDLDVIMLDKTGTITLTK	FTVTGIEILD			EAYQEEIILKYIGALEAHANPLAIGIM NYLKEK	
PHOSPHORYLATION DOMAIN							

PHOSPHORYLATION DOMAIN

FIG. 4 The amino-acid sequence of the ^{125}I -labelled peptides obtained from rabbit cardiac muscle SR ATPase and from dog fast-twitch muscle ATPase is shown aligned with homologous regions in other ATPases. (1) Rabbit slow-twitch muscle SR Ca^{2+} -ATPase²¹; (2) rabbit fast-twitch muscle SR Ca^{2+} -ATPase²²; (3) human plasma membrane Ca^{2+} -ATPase²³; (4) sheep plasma membrane Na^{+} + K^{+} ATPase²⁴; (5) torpedo plasma membrane

Na⁺+ K^{+} ATPase²⁵; (6) *Saccharomyces cerevisiae* H⁺-ATPase²⁶; (7) *Neorospira crassa* H⁺-ATPase²⁷; (8) *Escherichia coli* K⁺-ATPase²⁸, and (9) *Streptococcus faecalis* K⁺-ATPase²⁹. Despite the high degree of homology found in all ATPases around the phosphorylation domain, the PLN-binding region is unique to the two isoforms of the SR Ca^{2+} pump and is not even found in the plasma membrane (PM) Ca^{2+} -ATPase.

METHODS. The SR ATPase was crosslinked to non-phosphorylated, Denny-Jaffe-conjugated PLN as described in the legend to Fig. 1. Medium was supplemented with 0.5 mM EGTA and different amounts of CaCl_2 to give the calculated free Ca^{2+} concentration. Calcium dependence of ATPase activity was measured under the same conditions, but in the absence of modified PLN, using a coupled enzyme assay²⁰.



The domain of interaction of the ATPase with PLN has been identified. Crosslinked ^{125}I -labelled PLN-ATPase complex was cleaved at the azo linkage of the Denny-Jaffe reagent using dithionite. This removed PLN from its binding site while the ^{125}I label remained attached to the ATPase. After removal of PLN by extensive dialysis, cyanogen bromide digestion of the ATPase gave two peptides, both carrying the ^{125}I label, which must therefore have contained the binding domain. The peptides were purified (Fig. 3) and sequenced. Their molecular weights were identical, as judged from plasma desorption mass spectrometry, and their sequence was the same. They differed only in the position of labelling of the two neighbouring lysines (arrows in Fig. 4). Figure 4 shows that the peptide(s) originated from a region of the ATPase just C-terminal to the aspartyl residue that is phosphorylated during the transport cycle. Binding of unphosphorylated PLN to this site could therefore be interfering with the formation of the high-energy phosphorylated intermediate $\text{E}_1\text{-P}$, with its transition to the $\text{E}_2\text{-P}$ form, and/or with the hydrolysis of $\text{E}_2\text{-P}$, which is a rate-limiting step¹⁶.

The Ca^{2+} -ATPase of the SR shares a high degree of homology with all other ATPases of the phosphorylation type. In particular, the phosphorylation site is virtually identical in all these pumps (Fig. 4). Strikingly, however, the site of PLN interaction is a unique feature of the SR ATPase(s). Thus, regulation by PLN of ATPase activity, other than in the SR type, would not be possible. The PLN-binding domain is also found in the fast-twitch muscle isoform of the SR Ca^{2+} -ATPase (Fig. 4), so it has been possible also to crosslink PLN to ATPase preparations isolated from dog and rabbit fast skeletal muscle SR. The same lysine residues were labelled as in the slow-twitch/cardiac enzyme. Independent experiments using PLN affinity column chromatography showed that ATPase from rabbit fast skeletal muscle SR bound to the column only when the ATPase was in the E_2 form (data not shown). Evidently the regulation of the Ca^{2+} -pumping activity in fast- and slow-twitch muscle SR depends on the differential expression of the regulatory protein PLN, rather than on intrinsic structural differences in the ATPase isoforms present in the two muscle types. □

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1. Fujii, J. *et al.* *J. clin. Invest.* **79**, 301–304 (1987).
2. Wegener, A. D., Simmerman, H. K., Liepniesks, J. & Jones, L. R. *J. biol. Chem.* **261**, 5154–5159 (1986).
3. Lindemann, J. P., Jones, L. R., Hathaway, D. R., Henry, B. G. & Watanabe, A. M. *J. biol. Chem.* **258**, 464–471 (1983).
4. Garvey, J. L., Kranias, E. G. & Solaro, R. J. *Biochem. J.* **249**, 709–714 (1988).
5. Inui, M., Kadoma, M. & Tada, M. *J. biol. Chem.* **260**, 3706–3715 (1985).
6. Denny, J. B. & Blobel, G. *Proc. natn. Acad. Sci. U.S.A.* **81**, 5286–5290 (1984).
7. Inui, M., Chamberlain, B. K., Saito, A. & Fleischer, S. *J. biol. Chem.* **261**, 1794–1800 (1986).
8. Suzuki, T. & Wang, J. H. *J. biol. Chem.* **261**, 7018–7023 (1986).
9. Kirchberger, M. A., Borchman, D. & Kasinathan, C. *Biochemistry* **25**, 5484–5492 (1986).
10. Dupont, Y. *Biochim. biophys. Acta* **688**, 75–87 (1982).
11. Inesi, G. A. *Rev. Physiol.* **47**, 573–601 (1985).
12. Tada, M. *et al.* *J. biol. Chem.* **255**, 1985–1992 (1980).
13. Tada, M., Kirchberger, M. A. & Katz, A. M. *J. biol. Chem.* **250**, 2640–2647 (1975).

14. Tada, M. & Katz, A. M. *A. Rev. Physiol.* **44**, 401–423 (1982).
15. Chiesi, M. & Schwallier, R. *FEBS Lett.* **244**, 241–244 (1989).
16. Tada, M., Ohmori, F., Yamada, M. & Abe, H. *J. biol. Chem.* **254**, 319–326 (1979).
17. James, P. *et al.* *J. biol. Chem.* **263**, 2905–2910 (1988).
18. Van Winkle, W. B., Pitts, B. J. R. & Entman, M. L. *J. biol. Chem.* **253**, 8671–8673 (1978).
19. Laemmli, U. K. *Nature* **227**, 680–685 (1970).
20. Neet, K. E. & Green, N. M. *Arch. Biochem. Biophys.* **178**, 588–597 (1977).
21. MacLennan, D. H., Brandl, C. J., Korkzak, B. & Green, N. M. *Nature* **316**, 696–700 (1985).
22. Brandl, C. J., Green, N. M., Korkzak, B. & MacLennan, D. H. *Cell* **44**, 595–607 (1986).
23. Verma, A. K. *et al.* *J. biol. Chem.* **263**, 14152–14159 (1988).
24. Shull, G. E., Schwarz, A. & Lingrel, J. B. *Nature* **316**, 691–695 (1985).
25. Kawakami, K. *et al.* *Nature* **316**, 733–736 (1985).
26. Serrano, R., Kiehlbrandt, M. C. & Fink, G. R. *Nature* **319**, 689–693 (1986).
27. Addison, R. *J. biol. Chem.* **261**, 14896–14901 (1986).
28. Hesse, J. *et al.* *Proc. natn. Acad. Sci. U.S.A.* **81**, 4746–4750 (1984).
29. Solioz, M., Matthews, S. & Fürst, P. *J. biol. Chem.* **262**, 7358–7362 (1987).

Yeast replication factor-A functions in the unwinding of the SV40 origin of DNA replication

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CELL-free replication systems for simian virus 40 (SV40) DNA are taken to be a model for the replication of eukaryotic chromosomes, because only one viral protein is required to supplement the replication proteins provided by a human cell extract. To prove that these cellular proteins function in chromosomal DNA replication we have begun to identify homologous proteins in an organism that can be genetically manipulated. Here we report the identification of yeast replication factor-A (yRF-A) from *Saccharomyces cerevisiae* and show that it is functionally and structurally related to a human protein that is required for the initiation and elongation of SV40 DNA replication. Yeast RF-A, a multi-subunit phosphoprotein, is similar to the human protein in its chromatographic behaviour, subunit structure and DNA-binding activity. The yeast protein will fully substitute for the human protein in an early stage of the initiation of SV40 DNA replication. Substitution of yRF-A in the complete SV40 replication system, however, results in reduced DNA replication, presumably due to a requirement for species-specific interactions between yeast RF-A and the DNA polymerase complex.

Human cell extracts that are supplemented with purified SV40 large tumour antigen (T-Ag) support replication of plasmid DNAs containing SV40 origin of replication sequences^{1–3}. After biochemical fractionation, the crude extract has been replaced

by five purified proteins and one partially purified fraction (refs. 4–7, see ref. 8 for a review). One of these proteins, replication factor-A (RF-A), a multi-subunit protein with single-strand DNA (ssDNA)-binding activity^{9–11}, is required for the initiation of DNA replication by participating in the formation of an 'unwound complex' on the SV40 DNA template^{5,12–14}. This complex is an early intermediate in the initiation reaction and accumulates in the absence of dNTP precursors. It is detected by the accumulation of a topologically underwound plasmid DNA (form U) after phenol extraction and agarose gel electrophoresis. Form U DNA can be obtained in the absence of DNA synthesis by incubation of plasmid DNA containing the SV40 origin of replication with three purified components: T-Ag, topoisomerase I and RF-A. After the removal of the proteins, the unwound DNA is subjected to electrophoresis in an agarose gel in which form U DNA migrates slightly slower than form I DNA. This 'unwinding' assay was used to screen yeast extracts for components with an activity able to replace that of human RF-A. This assay was expected to detect heterologous ssDNA-binding proteins because the *Escherichia coli* ssDNA-binding protein (SSB), which functions during the initiation and elongation of *E. coli* DNA replication, substitutes for human RF-A in this assay^{15,16}.

Crude yeast cell extracts were unable to substitute for RF-A in the unwinding assay. An unwinding activity, however, could be detected in one fraction after chromatography of the extract on a ssDNA cellulose column. The active fraction bound tightly to ssDNA cellulose, requiring 1.5 M NaCl and 50% ethylene glycol for elution, similar to the conditions that elute human RF-A. The activity was further purified and found to co-fractionate on a Mono Q column with three polypeptides of relative molecular mass (M_r) 69,000 (69K), 36K and 13K (p69, p36 and p13; Fig. 1a and b). The M_r of these polypeptides closely

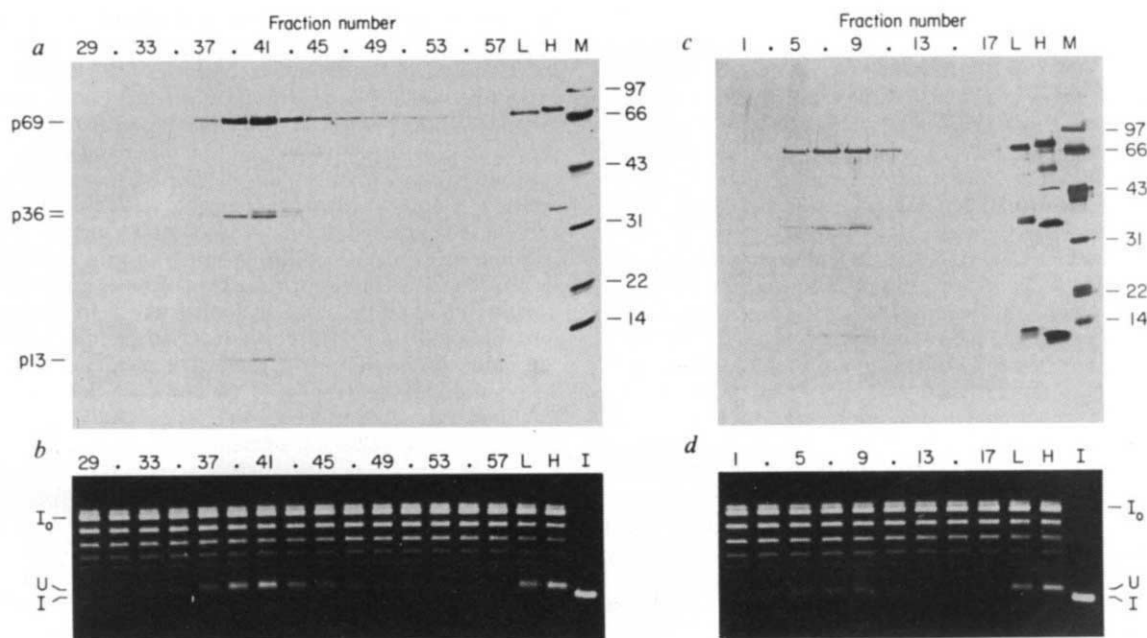


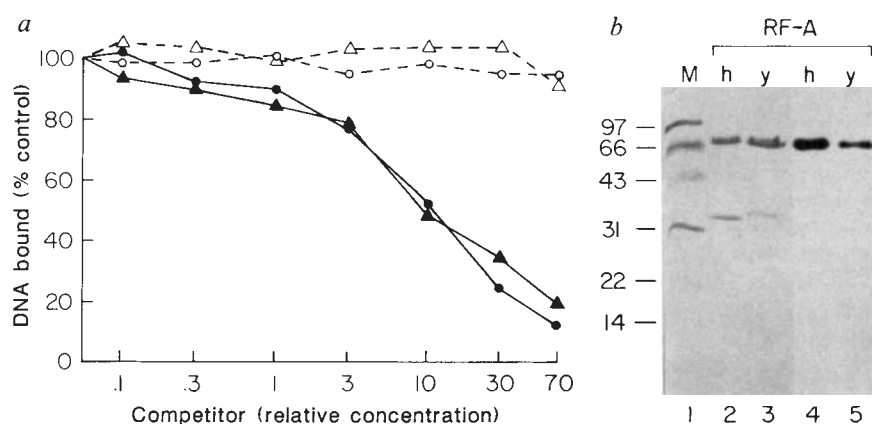
FIG. 1 Co-fractionation of unwinding activity and yRF-A. *a*, SDS-PAGE (17%) and Coomassie-brilliant-blue-stain analysis of yRF-A fractions from a Mono Q column. The fractions shown represent the portion of the elution gradient from 115 to 360 mM NaCl. *b*, Unwinding assays across Mono Q. *c*, SDS-PAGE (17%) and silver-stain analysis of fractions from a glycerol gradient sedimentation of yRF-A. The fractions shown represent the entire gradient (18 fractions total). *d*, Unwinding assays across the glycerol gradient. Lanes: L, load of each fractionation step; H, sample of human RF-A¹⁰; M, relative molecular mass markers ($M_r \times 10^{-3}$). I₀, form I relaxed DNA; U, form U unwound DNA; I, form I DNA.

METHODS. Strain BJ926 (10 litres) were grown and lysed as previously described¹⁸, except that the cells (160 g) were resuspended in 340 ml buffer A (25 mM Tris, pH 7.5, 1 mM EDTA, 10% glycerol, 1 mM DTT, 0.1 mM phenylmethylsulphonyl fluoride) with protease inhibitors (10 mM benzamidine, 100 $\mu\text{g ml}^{-1}$ bacitracin, 1 $\mu\text{g ml}^{-1}$ pepstatin A, 10 mM sodium bisulphite) and 1 M NaCl. The lysate was adjusted to 1 M NaCl, kept at 0 °C for 30 min, and centrifuged in a GSA rotor at 10,000 r.p.m. (16,200g at r_{max}) for 45 min. The supernatant (8.8 g protein) was adjusted to 0.5 M NaCl with buffer A and applied to a 2.5 $\times$ 8-cm Affi-gel Blue column. The column was washed

with two volumes each of buffer A containing 0.5 M NaCl, 0.8 M NaCl, and 2.5 M NaCl with 40% ethylene glycol. Peak protein fractions from the 2.5 M NaCl-ethylene glycol wash were pooled (350 mg protein), diluted to 0.5 M NaCl with buffer A and applied to a 2.5 $\times$ 6-cm ssDNA cellulose (USBC) column. This column was washed with two volumes each of buffer A containing 0.5 M NaCl, 0.75 M NaCl, and 1.5 M NaCl with 50% ethylene glycol. Peak protein fractions from the 1.5 M NaCl-ethylene glycol wash were pooled and dialysed against buffer A containing 200 mM NaCl and 20% sucrose. After clarification, this material (2.3 mg protein) was diluted to 100 mM NaCl with buffer A and applied to a Mono Q column (HR5/5). The column was washed with 4 ml buffer A containing 100 mM NaCl and eluted with a 10 ml linear gradient from 0.1 to 0.4 M NaCl. Fractions from this gradient (180–280 mM NaCl; peak, 220 mM NaCl) were pooled and dialysed against buffer A containing 25 mM NaCl and 20% sucrose. This procedure yielded 300 μg yRF-A at greater than 95% purity. Unwinding assays were performed as previously described⁵. Yeast RF-A (40 μg), Mono Q pool, was adjusted to 0.1 M NaCl and separated on a 5-ml, 15–35% glycerol gradient in buffer A with 0.1 M NaCl (SW50.1 rotor, 49,000 r.p.m. (300,000g at r_{max}), 21 h)⁴.

FIG. 2 Single-strand DNA-binding activity of yRF-A.

a, Competition of yRF-A binding to labelled ssDNA with double and single strand DNA. Human RF-A (triangles) or yRF-A (circles) was incubated with denatured ³²P-labelled pUC118 DNA in the presence of increasing amounts of double-strand pUC118 DNA (---) or single-stranded pUC118 DNA (—). *b*, Protein blot probed with ³²P-labelled DNA. Lanes: 1, marker proteins; 2, 5 μg human RF-A; 3, 5 μg yRF-A; 4, 0.5 μg human RF-A; and 5, 0.5 μg yRF-A. In lanes 1–3, the nitrocellulose blot was stained with amido black to detect transferred proteins. In lanes 4 and 5, the nitrocellulose blot was probed with ³²P-labelled single strand pBR322 DNA. M, M_r markers ($M_r \times 10^{-3}$); h, human; y, yeast. **METHODS.** *a*, Either hRF-A¹⁰ or yRF-A (Mono Q pool) (50 ng) were incubated for 30 min at 0 °C with various amounts of unlabelled competitor DNA and 15,000 c.p.m. (5 ng) pUC118 DNA that had been digested with *Hind*III, filled-in with [α -³²P]TTP and Klenow fragment enzyme, and thermally denatured. Competitor DNA consisted of pUC118 DNA digested with *Sma*I either before (double strand) or after (single strand) thermal denaturation. After the incubation, the samples were filtered through alkalai-washed nitrocellulose¹⁹



and counted. *b*, Proteins were separated by SDS-PAGE (17%), transferred to nitrocellulose, and either stained with amido black or probed⁶ with thermally denatured pBR322 DNA that had been digested with *Hpa*II and filled-in with [α -³²P]dCTP and [α -³²P]dGTP.

resemble those of human RF-A (70K, 34K and 11K; Fig. 1a and 1c, lane H). Note that the yeast p36 subunit appeared as a doublet on SDS-PAGE (see below). Glycerol gradient sedimentation analysis showed that these polypeptides co-sedimented with the unwinding activity (Fig. 1c and d) and, as reported for human RF-A¹⁰, the sedimentation of these polypeptides was unaffected by prior treatment with 6M urea (data not shown). These polypeptides therefore form a very stable complex.

Human RF-A binds to ssDNA in preference to double stranded DNA (dsDNA)^{6,10}. As a result of different techniques for measuring the affinity of RF-A for DNA, estimates of the degree of preferential binding range from 10-fold¹⁰ to 1,000-fold⁶. The nucleic-acid-binding activity of the yeast protein was characterized by using the more sensitive 'competition' filter binding assay⁶. Figure 2a shows that the binding of the protein to ssDNA was at least two orders of magnitude more sensitive to competition by ssDNA than by dsDNA. By using a similar analysis, we found that the binding of the protein to single-strand nucleic acid was specific for DNA; the affinity of the protein for poly(dA) was four orders of magnitude greater than its affinity for poly(A) (data not shown).

The identity of the DNA-binding subunit was determined by blotting gel-separated subunits to a nitrocellulose filter and probing with ³²P-labelled ssDNA. This assay indicated that the largest subunit of the yeast protein, p69, has strong DNA-binding activity, as does the largest subunit subunit of the human protein, p70 (ref. 6, Fig. 2b). On the basis of its apparent M_r estimated by SDS-PAGE, this protein complex does not seem to be similar to any previously characterized ssDNA-binding protein of yeast. Because of the conservation of unwinding activity, protein subunit structure and DNA-binding activity, we have termed the protein yeast replication factor-A (yRF-A).

One apparent difference between yRF-A and human RF-A (hRF-A) is the doublet appearance of the yeast p36 subunit on SDS-PAGE. As shown in Fig. 1a, the upper band of the p36 doublet bound more tightly to an anion exchange column (Mono Q), as would be expected for a more negatively charged species.

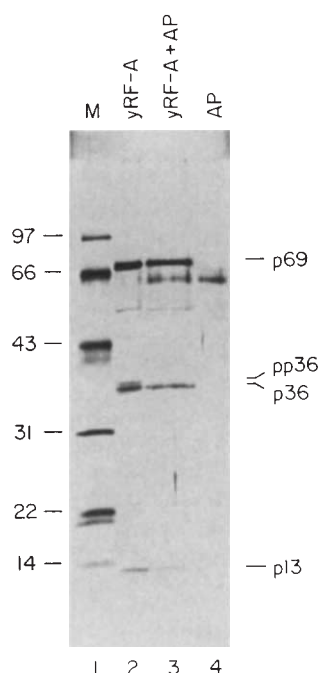


FIG. 3 Yeast RF-A is a phosphoprotein. Purified yRF-A (200 ng, Mono Q pool) was incubated with or without 0.1 U calf intestinal alkaline phosphatase (AP) at 37 °C for 60 min and subjected to SDS-PAGE (17%) and silver staining. Lanes: 1, marker proteins; 2, yRF-A alone; 3, yRF-A and alkaline phosphatase; 4, alkaline phosphatase. M, M_r markers ($M_r \times 10^{-3}$).

It is interesting to note that the p34 subunit of human RF-A undergoes a cell-cycle-regulated phosphorylation (S. Din, M. Fairman and B. S., unpublished result). We therefore investigated whether the yeast p36 doublet was the result of phosphorylation. Alkaline phosphatase treatment of yRF-A resulted in the disappearance of the upper p36 doublet band consistent with this band representing a phosphorylated species (Fig. 3). We are now testing whether the phosphorylation of the yeast p36 subunit is controlled during progression of the cell cycle.

Yeast RF-A was tested for its ability to substitute for human RF-A in the SV40 replication reaction reconstituted with purified proteins⁵. Saturating levels of human RF-A in the replication reaction yielded an incorporation of 40 pmol dAMP, whereas saturating levels of yRF-A yielded a maximum synthesis of 2.5 pmol dAMP, and higher levels of yRF-A than human RF-A inhibited the reaction (Fig. 4a). Thus, unlike the initiation reaction, yRF-A could not efficiently substitute for human RF-A

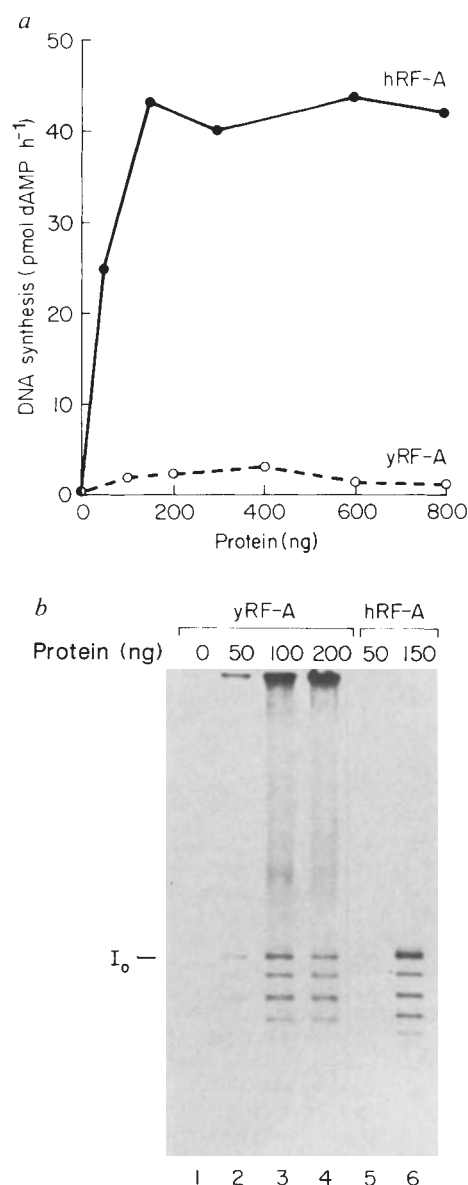


FIG. 4 Yeast RF-A substitution in SV40 replication. Various amounts of protein were incubated with optimized amounts of replication factors, T-Ag, topoisomerase I, topoisomerase II, PCNA, RF-C, and fraction IIA at 37 °C for 60 min, and subjected to TCA precipitation (a) or agarose gel electrophoresis (b) as previously described⁵. For the autoradiogram shown in b, the exposure time of lanes 1-4 was ten times that of lanes 5 and 6. Human RF-A, hRF-A; I_0 form I relaxed DNA.

in the complete replication reaction, although analysis of the small amount of replication products by agarose gel electrophoresis revealed topoisomers consistent with completely replicated molecules, similar to those obtained with human RF-A (Fig. 4b).

Although yRF-A seems to fully substitute in the unwinding reaction, the ability of yRF-A to fully substitute in the complete replication reaction could depend on an additional function of RF-A. We have recently shown that hRF-A stimulates both DNA polymerases α and δ , implicating a role for human RF-A in the elongation stage of DNA replication²⁰. Preliminary results indicate that yRF-A does not stimulate human DNA polymerase α . It is probable that yRF-A, therefore, cannot efficiently substitute in the priming and elongation stages of SV40 DNA replication, because of a requirement for a species-specific interaction between RF-A and the priming-polymerase complex.

The divergence of yeast and human RF-A is confirmed by the failure of both polyclonal and monoclonal antibodies, raised against human RF-A, to recognize yRF-A. More recently, polyclonal antibodies have been raised against the individual subunits of yRF-A and they fail to bind to human RF-A (unpublished observation). This situation is similar to that involving the proliferating cell nuclear antigen (PCNA), another human cell replication factor that is required for SV40 DNA replication *in vitro*⁸, and its yeast analogue yPCNA. PCNA, from human and yeast sources, functions as an accessory protein for DNA polymerase δ and yeast DNA polymerase III, respectively. Yeast PCNA fails to cross-react with antibodies raised against the mammalian protein and although yPCNA stimulates DNA polymerase δ , it does so poorly; the level of yPCNA required is 10-fold higher than the level of human PCNA required for stimulation¹⁷.

The combined use of a mammalian cell-free system with biochemical and genetic analysis of yeast should provide an insight into eukaryotic chromosomal DNA replication. A similar approach has recently been used for understanding other complex macromolecular processes, such as transcription and protein transport in eukaryotic cells. The isolation of a ssDNA-binding protein from yeast that probably functions at the initiation stage of DNA replication will be a valuable entry point for understanding the mechanism of initiation of chromosomal DNA replication. Because this event is highly regulated during the eukaryotic cell cycle, the components involved in the initiation of DNA replication provide attractive targets for growth regulatory processes. The identification of yRF-A as a phosphoprotein should enable a biochemical and genetic analysis of those controls. □

Induction of the *Escherichia coli* lactose operon selectively increases repair of its transcribed DNA strand

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NUCLEOTIDE excision repair helps to ameliorate the lethal and mutagenic consequences of DNA damage by removing helix-distorting lesions from cellular genomes¹. We have previously analysed the removal of ultraviolet-induced cyclobutane pyrimidine dimers from specific DNA sequences in mammalian cells and demonstrated that transcriptionally active genes are preferentially repaired²⁻⁴. Additionally, we found that in rodent and human cells only the transcribed strand of the dihydrofolate reductase gene is selectively repaired⁵. Transcription is blocked by pyrimidine dimers in template DNA⁶ and the selective removal of these lesions seems to be important for cell survival after irradiation with ultraviolet light^{2,7,8}. To determine whether this feature of repair is common to prokaryotes and eukaryotes and better to understand its mechanism, we have investigated repair in the two separate DNA strands of the lactose operon of ultraviolet-irradiated *Escherichia coli*. We find a dramatic difference in the repair of the two strands only when transcription is induced. Most dimers are removed from the transcribed strand of the induced operon within five minutes of irradiation. In the non-transcribed strand, repair is significantly slower and resembles that found in both strands of the uninduced operon. Thus there seems to be a mechanism that couples nucleotide excision repair and transcription.

The lactose operon of *E. coli* is well characterized and its transcription can be modulated⁹. It contains three genes in a single transcription unit, *lacZ*, *lacY* and *lacA*, which encode β -galactosidase, galactoside permease and thiogalactoside transacetylase, respectively. Synthesis of the polycistronic message is induced by β -galactosides such as lactose or isopropyl- β -D-thiogalactoside (IPTG). In the absence of inducer, initiation of transcription is very infrequent.

Using our earlier methodology^{5,10}, we measured the removal of pyrimidine dimers from the respective DNA strands of specific restriction fragments of the *lac* operon in *E. coli* K12 cells grown in the presence or absence of IPTG. After irradiation with ultraviolet light, culture samples were collected immediately to determine the initial frequency of dimers, and then further samples were taken after incubating for increasing periods of time to allow repair. High molecular weight DNA was purified and treated with restriction enzymes. Only unreplicated DNA was examined to avoid complication by dimer-free DNA generated by replication, which would cause an overestimate of the amount of repair. Two aliquots of each DNA sample were analysed, one of which was treated with T4 endonuclease V to produce a single-strand break specifically at each cyclobutane pyrimidine dimer¹¹. Samples were electrophoresed in parallel under denaturing conditions and the DNA transferred to a support membrane. These Southern blots were then hybridized with ³²P-labelled strand-specific RNA probes to quantitate the full-length restriction fragments at each time point. As repair occurs, fewer T4 endonuclease-sensitive sites remain in the DNA, resulting in fewer strand breaks by endonuclease and more full-length restriction fragments in the enzyme-treated sample. The average number of dimers per fragment was

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- Li, J. J. & Kelly, T. J. *Proc. natn. Acad. Sci. U.S.A.* **81**, 6973-6977 (1984).
- Stillman, B. W. & Gluzman, Y. *Molec. cell. Biol.* **5**, 2051-2060 (1985).
- Wobbe, C. R., Dean, F., Weissbach, L. & Hurwitz, J. *Proc. natn. Acad. Sci. U.S.A.* **82**, 5710-5714 (1985).
- Tsurimoto, T. & Stillman, B. *Molec. cell. Biol.* **9**, 609-619 (1989).
- Tsurimoto, T., Fairman, M. P. & Stillman, B. W. *Molec. cell. Biol.* **9**, 3839-3849 (1989).
- Wold, M. S., Weinberg, D. H., Virshup, D. M., Li, J. J. & Kelly, T. J. *J. biol. Chem.* **264**, 2801-2809 (1989).
- Ishimi, Y., Claude, A., Bullock, P. & Hurwitz, J. *J. biol. Chem.* **263**, 19723-19733 (1988).
- Stillman, B. A. *Rev. Cell. Biol.* **5**, 197-245 (1989).
- Wobbe, C. R. *et al. Proc. natn. Acad. Sci. U.S.A.* **84**, 1838-1838 (1987).
- Fairman, M. P. & Stillman, B. *EMBO J.* **7**, 1211-1218 (1988).
- Wold, M. S. & Kelly, T. J. *Proc. natn. Acad. Sci. U.S.A.* **85**, 2523-2527 (1988).
- Dean, F. B. *et al. Proc. natn. Acad. Sci. U.S.A.* **84**, 16-20 (1987).
- Dodson, M., Dean, F. B., Bullock, P., Echols, H. & Hurwitz, J. *Science* **238**, 964-967 (1987).
- Wold, M. S., Li, J. J. & Kelly, T. J. *Proc. natn. Acad. Sci. U.S.A.* **84**, 3643-3647 (1987).
- Dean, F. B. *et al. Proc. natn. Acad. Sci. U.S.A.* **84**, 16-20 (1987).
- Wold, M. S., Li, J. J. & Kelly, T. J. *Proc. natn. Acad. Sci. U.S.A.* **84**, 3643-3647 (1987).
- Bauer, G. A. & Burgers, P. M. J. *Proc. natn. Acad. Sci. U.S.A.* **85**, 7506-7510 (1988).
- Bauer, G. A., Heller, H. M. & Burgers, P. M. J. *J. biol. Chem.* **263**, 917-924 (1988).
- McEntee, K., Weinstock, G. M. & Lehman, I. R. *Proc. natn. Acad. Sci. U.S.A.* **77**, 857-861 (1980).
- Tsurimoto, T. & Stillman, B. *EMBO J.* **8** (in the press).

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calculated from the ratio of full-length restriction fragments in the enzyme-treated and untreated samples, using the Poisson expression.

Two restriction fragments were examined: the 6.6 kilobase (kb) *Apa*I–*Sst*II fragment that contains 1.5 kb of sequence outside the coding region of the operon, and the 5.2 kb *Nar*I–*Nru*I restriction fragment that resides almost entirely within the transcription unit (Fig. 1a). Strand-specific RNA probes (Fig. 1b) were obtained by subcloning a fragment of the *lacZ* gene into the multiple cloning site of the vector pGEM-3Z. The construct, pZH10, contains the *lacZ* sequence flanked by two different phage RNA polymerase promoters oriented in opposite directions. Linearizing the plasmid with *Eco*RI and incubating with SP6 RNA polymerase generates an RNA probe specific for the transcribed strand of the operon, whereas linearizing with *Bam*HI and incubating with T7 RNA polymerase generates an RNA probe specific for the non-transcribed strand.

Repair was examined in the 6.6 kb *Apa*I–*Sst*II fragment of the uninduced or induced operon at 2.5, 5, 10 and 20 min after irradiation with ultraviolet light (40 J m^{-2}) (Fig. 2). Levels of β -galactosidase were 4,800 units and 11 units¹² in cultures grown in the presence or absence of IPTG. Southern blots were hybridized with the probe specific for the transcribed strand, that probe was removed and the blot was then hybridized with the probe specific for the non-transcribed strand. The initial level of damage was the same in each strand, whether or not transcription had been induced. Visual comparison of the enzyme-treated samples probed for the two different strands at each repair time point reveals similar amounts of full-length restriction fragments when the operon was not induced, but significantly more full-length fragments for the transcribed strand when the operon was induced. Quantitation indicates that there is a dramatic difference in the rate of repair in the transcribed and non-transcribed strands of the operon only when transcription was induced with IPTG (Fig. 3). Within 5 min, most dimers were removed from the transcribed strand of the induced operon, whereas little repair was detected at this time in the non-transcribed strand or in either strand of the uninduced operon. The

repair rates in each strand of the uninduced operon and in the non-transcribed strand of the induced operon were comparable. Repair was almost complete within 40 min after irradiation (data not shown).

This study provides two important observations: (1) Selective removal of pyrimidine dimers from the transcribed strand of an active gene is not unique to mammalian or even eukaryotic excision repair. Repair in the induced *lac* operon of ultraviolet-irradiated *E. coli* resembles repair in the active dihydrofolate reductase (*DHFR*) gene of ultraviolet-irradiated human cells⁵: repair in the transcribed strand is significantly faster than that in the non-transcribed strand, but most dimers are removed from both strands within one generation period in the respective cell types. (2) This selective removal of pyrimidine dimers from the transcribed strand of a gene is abolished in the absence of significant levels of transcription.

Bockrath and Palmer¹³ studying ultraviolet-irradiated *E. coli* noted a more rapid decline in mutations generated by lesions that were deduced to be in the transcribed strand of a glutamine transfer RNA gene than in mutations generated by lesions in the non-transcribed strand of the gene. This difference in mutation frequency did not occur in a mutant deficient in nucleotide excision repair. They suggested that selective repair of the transcribed strands of the ⁶IntRNA genes might account for the observed decline in mutation frequency, a notion strengthened by the demonstration that the strand-specific loss of mutations is unrelated to the polarity of replication¹⁴. We have been unable to analyse repair in the respective strands of the ⁶IntRNA genes (unpublished data) because the method described here requires very high doses of ultraviolet light to examine small sequences. A similar strand bias in mutation frequency was noted by Bockrath *et al.*¹⁵ using ethyl methanesulphonate, and Reed and Hutchinson¹⁶ have reported a large bias in mutation frequency towards the non-transcribed strand of the λ *cl* gene in *E. coli* treated with another alkylating agent, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine. It will be important to determine whether differences in the removal of DNA damage from the respective strands of active genes in *E. coli* and mammalian cells correlate

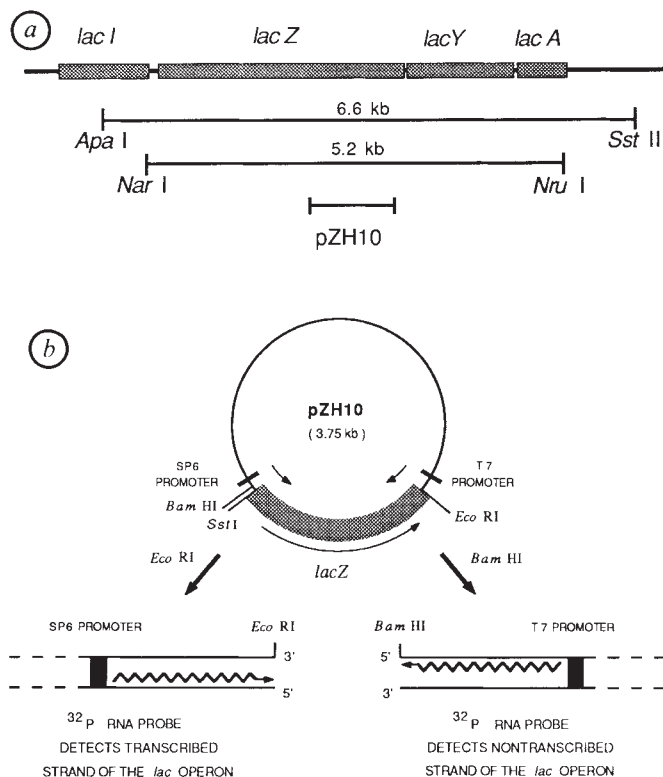
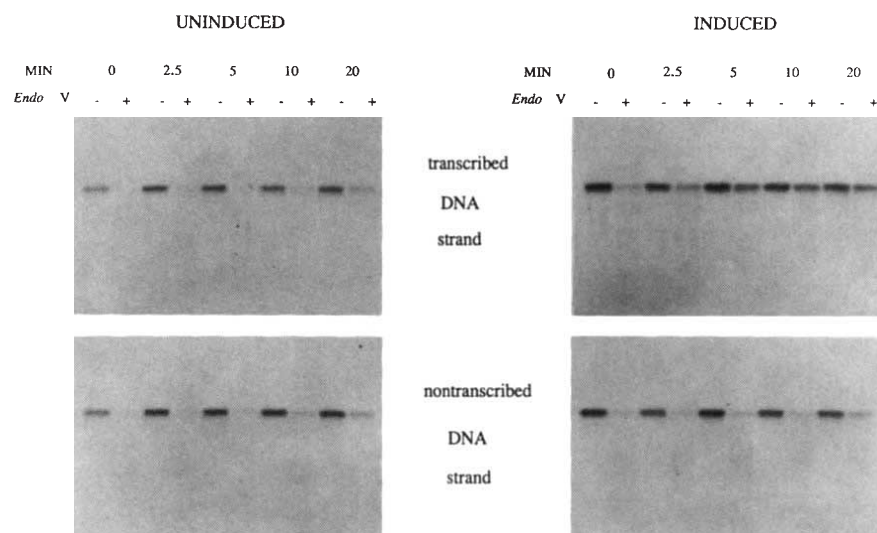


FIG. 1 a, Map of *E. coli* lactose operon. The positions of the genes within the operon, *lacI*, the *lacZ* insert in pZH10 used to generate probes, and relevant restriction enzyme sites are shown. b, Illustration of the plasmid pZH10 and application to generate strand-specific RNA probes. Plasmid pZH10 contains 1 kb of the *E. coli lacZ* gene (thick arc) and the vector sequences from pGEM-3Z (thin line), which include the SP6 and T7 RNA polymerase promoters (heavy bars). The directions of transcription of the *lacZ* gene *in vivo* and the SP6 and T7 promoters are indicated by arrows around the plasmid diagram. Strand-specific RNA probes are indicated by wavy lines.

METHODS. Restriction enzyme sites in the *lac* operon region were obtained from sequence data (accession numbers: J01636, J01637, K01483, K01793) in the GenBank Data Bank²⁷ (release 59) using the Bionet resources (Intelligenetics). The plasmid pZH10 was constructed by isolating a 1 kb *Eco*RI–*Sst*I *lacZ* fragment from the plasmid pZH1 (ref. 28) and subcloning it into the *Eco*RI–*Sst*I sites of pGEM-3Z. Cultures of DH5alpha (BRL) were transformed with the ligation products, and recombinants were selected as white colonies on LB plates supplemented with $100 \mu\text{g ml}^{-1}$ ampicillin, 0.5 mM IPTG, and $40 \mu\text{g ml}^{-1}$ X-Gal. Plasmids were isolated and purified by two consecutive bandings in CsCl/ethidium bromide gradients. Plasmid DNA was digested with *Bam*HI (*Sst*I generates 3' protruding ends) or *Eco*RI and RNA probes were prepared as previously described⁵.

FIG. 2 Autoradiograph illustrating repair in the *lac* operon when it is uninduced and induced. DNA was isolated at the times indicated above the lanes, restricted with *Apal* and *SstII*, and not treated (–) or treated (+) with T4 endonuclease V. Samples (0.5 µg) of DNA were loaded. ³²P-labelled RNA probes were made from pZH10.

METHODS. Cells were grown, irradiated with ultraviolet light, and incubated as previously described²⁹, with some modifications. Cultures of *E. coli* K-12 strain SR108 (*F*[–]λ^s*thyA deo*[–]) (provided by A. Ganesan) were grown at 37 °C in Difco Bacto Minimal Broth Davis, supplemented with 0.4% glucose, 0.05% casein hydrolysate, 2 µg ml^{–1} thymine and 0.5 µCi ml^{–1} [³H]thymine. Cultures were grown to a density of ~3 × 10⁷ cells per ml, collected on 0.22-µm pore size Millipore filters (GSPW) by filtration, and resuspended at the original density in unsupplemented Minimal Broth. Cultures were irradiated with 40 J per m² of ultraviolet light (254 nm) and incubated for the specified repair periods in Minimal Broth containing 0.4% glucose, 0.05% casein hydrolysate and 10 µg ml^{–1} 5-bromouracil. For studies of repair in the induced *lac* operon, IPTG (0.1 mM) was included in the medium before and after ultraviolet radiation. (Because glucose was present in the medium, the operon was not fully induced.) β-Galactosidase levels were assayed as previously described¹², except cells were permeabilized by freeze-thawing instead of by toluene treatment. High molecular weight DNA was isolated²⁹, treated with restriction enzymes, sedimented to equilibrium in CsCl density gradients to remove replicated DNA and treated with T4 endonuclease V



(ref. 5). Samples of DNA (0.5 µg) were treated with 1 µl T4 endonuclease V (incision activity of enzyme preparation, 5.5×10^{11} sites per µl min^{–1} at 37 °C). Samples were electrophoresed in 0.6% alkaline agarose gels and the DNA was transferred to a Genatran nylon membrane. The membrane was hybridized with ³²P-labelled probe, washed and exposed to X-ray film as described⁵. The amount of radioactivity hybridized to the fragment of interest was determined by scanning densitometry of the autoradiograph.

with mutation frequencies in these strands, but this examination may be complicated by other cellular processes that influence mutation frequency^{17–19}.

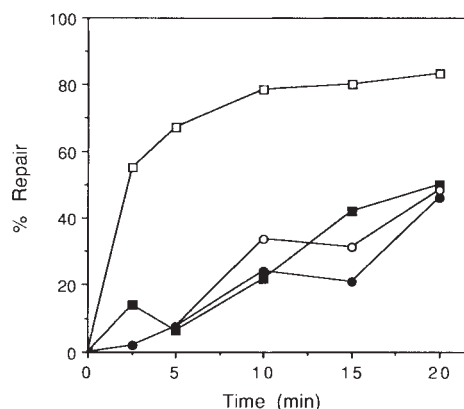
There is strong evidence that transcription is blocked by pyrimidine dimers in template DNA in both prokaryotic and eukaryotic cells⁶. Initiation occurs normally on the damaged template (assuming no damage in the initiation region) and chain elongation proceeds until a dimer is encountered, resulting in premature termination. It is likely that only damage to the template strand produces this effect; this has been directly shown *in vitro* for psoralen monoadducts²⁰. A repair mechanism that specifically detects transcription-blocking DNA damage should be advantageous to the cell if maintenance of constant levels of essential transcripts is critical for cell survival. As ultraviolet irradiation actually induces the transcription of a collection of genes²¹ while producing DNA lesions that block transcription elongation, the rapid removal of transcription-blocking lesions should facilitate the expression of such ultraviolet-induced genes. It is tempting to speculate that for some genes, the purpose of ultraviolet-induced transcription is not simply to provide a

gene product, but rather to ensure that DNA damage is rapidly removed from certain essential sequences.

The molecular details of nucleotide excision repair are more complicated and less well understood in mammalian cells than in *E. coli*. A larger number of genes are involved and although some have recently been cloned^{22,23}, their functions are unknown. It is also not clear how heterogeneities in the chromatin structure or organization of particular sequences in the complex mammalian genome affect DNA repair. Indeed, we originally suggested that differences in the repair of transcriptionally active and inactive sequences in mammalian cells are due to differences in their chromatin structure^{2,4}. Our finding that preferential repair of the *DHFR* gene in human and rodent cells is due to selective repair of only the transcribed strand of the gene challenged this model. The strand-specific repair in the *E. coli lac* operon indicates that features of eukaryotic chromatin structure are not required for selective removal of damage from active genes. Furthermore, as this feature of repair has been conserved in bacteria and mammalian cells, it is not something that just evolved in higher eukaryotes as a mechanism to deal

FIG. 3 The effect of transcription on pyrimidine dimer removal from the DNA strands of the *lac* operon. The average number of dimers was measured in the 6.6-kb *Apal*–*SstII* restriction fragment at 0, 2.5, 5, 10 and 20 min after irradiation (40 J m^{–2}) and in the 5.2-kb *NarI*–*NruI* restriction fragment at 0, 5, 10, 15 and 20 min after irradiation (40 J m^{–2}). Values at 5, 10 and 20 min are an average of results from the two fragments. T (open symbols), transcribed strand; NT (filled symbols), non-transcribed strand; squares, induced; circles, uninduced.

METHODS. The average number of fragment was calculated from the percentage of fragments that had no dimers ('zero class') by using the Poisson expression (number of dimers per fragment = $-\log_e$ of the zero class). The zero class is equal to the ratio of the amount of full-length fragments in the enzyme-treated and untreated samples.



with the organization of sequences within their complex genomes.

A comprehensive description of nucleotide excision repair has only been achieved for *E. coli*^{24,25}. Most, if not all, of the genes involved have been cloned and their products studied *in vitro*. At least six proteins are involved: UvrA, UvrB and UvrC, assembled in an 'excinuclease complex', and UvrD, DNA polymerase I and DNA ligase. It is thought that the distortion of the DNA helix produced by the pyrimidine dimer (or bulky DNA adducts), rather than the damage itself, is the substrate for the UvrABC complex. Two incisions are made in the DNA strand near the damage: the eighth phosphodiester bond 5' to, and the fourth or fifth phosphodiester 3' to a pyrimidine dimer are cleaved. The oligonucleotide containing the dimer is removed, a repair patch is synthesized using the complementary strand as a template, and ultimately the repair patch is ligated to the contiguous parental DNA strand. The strand-specificity in the repair of the induced *lac* operon demonstrated here indicates an association or coupling between transcription and nucleotide excision repair. Considering the ultraviolet-sensitive mutants that have been characterized in *E. coli*, it seems unlikely that the preferential repair of the transcribed strands of active genes is carried out by an entirely different and as-yet-uncharacterized repair system. It is likely that selectivity is imposed by targeting of the UvrABC complex to damage in the transcribed strand. There need not be a specific factor whose sole role is to mediate such targeting. For example, an RNA polymerase complex blocked at a lesion in the transcribed strand may itself facilitate recognition. This could be analogous to the mechanism whereby *E. coli* photolyase, an enzyme that specifically binds and photo-reverses pyrimidine dimers, actually stimulates the removal of dimers by the UvrABC excision nuclease²⁶. Studies using the genetic and biochemical tools available in *E. coli* are needed to test this model, which should enhance our understanding of transcription-associated DNA repair in both bacteria and mammalian cells. □

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1. Friedberg, E. C. in *DNA Repair* (Freeman, San Francisco, 1985).
2. Bohr, V. A., Smith, C. A., Okumoto, D. S. & Hanawalt, P. C. *Cell* **40**, 359-369 (1985).
3. Madhani, H. D., Bohr, V. A. & Hanawalt, P. C. *Cell* **45**, 417-423 (1986).
4. Mellon, I., Bohr, V. A., Smith, C. A. & Hanawalt, P. C. *Proc. natn. Acad. Sci. U.S.A.* **83**, 8878-8882 (1986).
5. Mellon, I., Spivak, G. & Hanawalt, P. C. *Cell* **51**, 241-249 (1988).
6. Sauerbier, W. & Hercules, K. A. *Rev. Genet.* **12**, 329-363 (1978).
7. Mayne, L. V. & Lehmann, A. R. *Cancer Res.* **42**, 1493-1498 (1982).
8. Mayne, L. V., Mullenders, L. H. F. & van Zeeland, A. A. in *Mechanisms and Consequences of DNA Damage Processing* (eds Friedberg, E. C. & Hanawalt, P. C.) 349-353 (Liss, New York, 1988).
9. *The Lactose Operon* (Cold Spring Harbor Laboratory, New York, 1970).
10. Bohr, V. A. & Okumoto, D. S. in *DNA Repair: A Laboratory Manual of Research Procedures* (eds Friedberg, E. C. & Hanawalt, P. C.) **3**, 347-366 (Dekker, New York, 1988).
11. Sancar, A. & Rupp, W. D. *Cell* **33**, 249-260 (1983).
12. Miller, J. H. in *Experiments in Molecular Genetics* 352-355 (Cold Spring Harbor Laboratory, Cold Spring Harbor, 1972).
13. Bockrath, R. C. & Palmer, J. E. *Molec. gen. Genet.* **156**, 133-140 (1977).
14. Engstrom, J., Larsen, S., Rogers, S. & Bockrath, R. C. *Mutation Res.* **132**, 143-152 (1984).
15. Bockrath, R. C., Barlow, A. & Engstrom, J. *Mutation Res.* **183**, 241-247 (1987).
16. Reed, J. & Hutchinson, F. *Molec. gen. Genet.* **208**, 446-449 (1987).
17. Vrieling, H. et al. *Molec. cell. Biol.* **9**, 1277-1283 (1989).
18. Herman, R. K. & Dworkin, N. B. *J. Bact.* **106**, 543-550 (1971).
19. Savić, D. J. & Kanazir, D. T. *Molec. gen. Genet.* **118**, 45-50 (1972).
20. Shi, Y. B., Gampert, H. & Hearst, J. E. *Nucleic Acids Res.* **15**, 6843-6854 (1987).
21. Fornace, A. J., Jr, Alamo, I., Jr, & Hollander, M. C. *Proc. natn. Acad. Sci. U.S.A.* **85**, 8800-8804 (1988).
22. Hoeijmakers, J. H. J. et al. in *Mechanisms and Consequences of DNA Damage Processing* (eds Friedberg, E. C. & Hanawalt, P. C.) 281-287 (Liss, New York, 1988).
23. Thompson, L. H., Weber, C. A. & Carrano, A. V. in *Mechanisms and Consequences of DNA Damage Processing* (eds Friedberg, E. C. & Hanawalt, P. C.) 289-293 (Liss, New York, 1988).
24. Sancar, A. & Sancar, G. W. A. *Rev. Biochem.* **57**, 29-67 (1988).
25. Grossman, L., Caron, P. R., Mazur, S. J. & Oh, E. Y. *Fedn Proc.* **2**, 2696-2701 (1988).
26. Sancar, A., Franklin, K. A. & Sancar, G. B. *Proc. natn. Acad. Sci. U.S.A.* **81**, 7397-7401 (1984).
27. Bilofsky, H. S. et al. *Nucleic Acids Res.* **14**, 1-4 (1986).
28. Ganesan, A. K. & Spivak, G. in *DNA Repair: A Laboratory Manual of Research Procedures* (eds Friedberg, E. C. & Hanawalt, P. C.) **3**, 295-310 (Dekker, New York, 1988).
29. Smith, C. A., Cooper, P. K. & Hanawalt, P. C. in *DNA Repair: A Laboratory Manual of Research Procedures* (eds Friedberg, E. C. & Hanawalt, P. C.) **1B**, 289-305 (Dekker, New York, 1981).

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Engineering monoclonal antibodies

J.D. Rodwell

Efforts to alter the mAb component of pharmaceuticals have focused on making them more human-like and smaller. Ultimately, the smallest molecular recognition unit derived from antibodies may be a single complementarity region.

MURINE monoclonal antibodies (mAbs) conjugated to drugs, isotopes, or toxins are being utilized as the targeting components of diagnostic and therapeutic agents for cancer, cardiovascular and other diseases. However, because of the limitations of administering nonhuman proteins, efforts to engineer new generations of antibodies, or antibody-like molecules, are already underway in both the biotechnology industry and in academe. These efforts are directed towards making the antibodies more human-like, smaller in size, or both (Fig. 1), with the aim of lowering the immunogenicity of these proteins in man and optimizing their target penetration and pharmacokinetic characteristics.

mAb immunogenicity

One way to decrease mAb immunogenicity, as described by Morrison and her colleagues¹, involves the development of 'chimaeric' antibody technology. Rearranged DNA encoding mouse variable (VH, VL) regions is fused to DNA encoding human constant heavy and light chains. On expression, interspecies antibody chimaeras are obtained, with the same antigen-binding specificity as the parent molecule. Because most of the protein comprises human amino acid sequences, these antibodies should be less antigenic. A recent report by LoBuglio *et al.*² suggests that this approach may, indeed, result in reduced immunogenicity. A chimaeric antibody derived from the mouse monoclonal 17-1A was administered to patients with metastatic colon cancer. Compared to the parent murine antibody, the chimaeric molecule was substantially less immunogenic, with only one in 10 patients developing an antibody response.

Another approach is CDR (complementarity determining regions) grafting, which was developed by Winter and his colleagues³. This technique involves incorporation of the CDRs of mAbs into an expression system encoding human variable and constant regions. As the resulting 'humanized' antibodies contain only a small fraction of the amino acid sequence of mouse origin, it was reasoned that there would be little problem with immunogenicity. In a preliminary communication⁴, Hale *et al.* reported remission in two non-Hodgkin lymphoma patients who received daily doses of 1–20 mg of a humanized mAb (CAMPATH-1-H) over 43 days. Significantly, neither patient

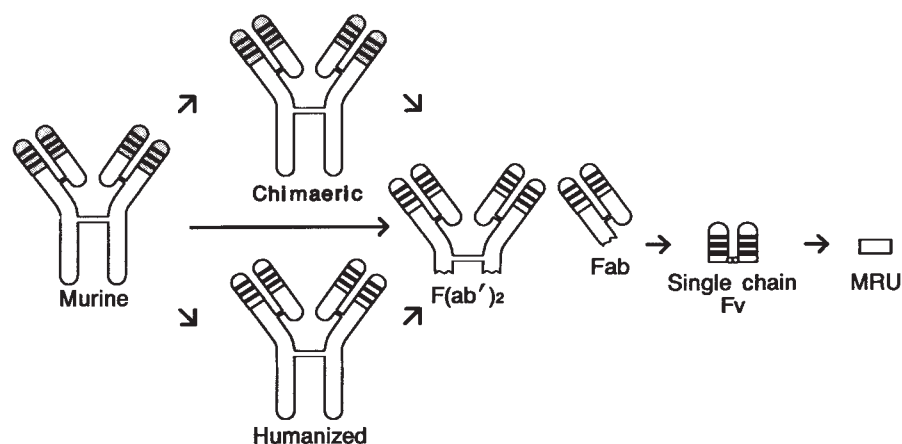


FIG. 1 Efforts to alter the mAb component of pharmaceuticals have led to the development of chimaeric, CDR-grafted (humanized) antibodies and to progressively smaller molecules.

developed a detectable immune response to the antibody.

Molecular size

While the above approaches may reduce patient sensitization to injected antibodies, they do not deal with the problem of molecular size. One potential solution is the use of antigen-binding fragments (Fabs), which penetrate target tissue better and clear from blood more rapidly than intact antibodies. As both chimaeric and humanized antibodies can be substrates for Fab production, Fabs produced in this way offer the same advantages over intact antibodies as mouse Fabs, while retaining lower immunogenicity. For diagnostic imaging of highly vascularized regions, such as thrombi, background clearance of labelled Fabs through the kidneys allows more rapid visualization of damaged myocardium. For tumour therapy, where the goal is to deliver a cytotoxic dose of an appropriate isotope, drug, or toxin to as many target cells as possible, Fabs penetrate tumours more effectively, producing a more even distribution⁵. The cytotoxic efficacy of therapeutic isotopes may be expected to rise with better and more uniform tumour penetration.

The underlying assumption that smaller molecular size is better has also been addressed by the preparation of antigen-binding Fv fragments and 'single-chain' antibodies. Fvs are specific variable regions pairs (VH and VL), and have been expressed in *Escherichia coli*⁶ and myeloma cells⁷. Single-chain antibodies are an interesting variation on this approach, in that VH and VL genes are joined together by a DNA segment encoding a peptide.

This peptide is capable of bridging the carboxy terminus of one V domain and the amino terminus of the other, resulting in an Fv-like molecule synthesized as one continuous amino acid polymer. This approach has been reported to yield a properly folded (and paired) VH-VL gene product with the same antigen-binding characteristics as the antibody from which it was derived^{8,9}.

Future directions

All of these new approaches promise to improve upon results achieved with intact murine mAbs, and so we are developing strategies for the clinical evaluation of each approach. However, simplification of the molecular recognition component of this new generation of pharmaceuticals will not end here. At least two independent groups have reported that peptides, each representing the sequence of a single CDR from a mAb, can mimic the specificity of the parent molecule.

Williams *et al.*¹⁰ have shown that a peptide with the sequence of the second CDR of the light chain of mAb 87.92.6, which binds to the reovirus type-3 receptor on cells, mimics the ability of the antibody to 'down-regulate' the receptor and inhibit DNA synthesis. Taub *et al.*¹¹ have described a peptide with the sequence of the third CDR of the heavy chain of PAC1 — an antibody specific for the GP IIb-IIIa complex on activated human platelets. Like the parent antibody, this peptide inhibits the binding of fibrinogen to platelets and also inhibits the binding of the PAC1 antibody itself. In both cases, the CDR peptides have homology with the sequence of the 'native' protein ligand.

Williams' peptide has sequence homology with the reovirus 3 haemagglutinin, and Taub's peptide has sequence homology with a region of fibrinogen that binds to the GP IIb-IIIa complex.

The CDRs of antibodies may, therefore, be an important source of amino-acid sequence information that would facilitate the preparation of small recognition elements. The molecular weights of these recognition elements would be as low as a few per cent of the weight of an IgG antibody. Consequently, these elements, conjugated or fused to diagnostic and therapeutic effector molecules, would constitute an important new class of self-targeting pharmaceuticals.

The clinical potential for the diagnosis and treatment of human diseases with self-targeting pharmaceuticals using mouse mAbs is finally being realized. Knowledge derived from the use of intact antibody conjugates will allow further innovation as each component (molecular recognition, effector) is better understood. We can expect new generation(s) of antibody-like recognition units to be engineered using molecular surgery to remove structures that have evolved for functions secondary to antigen binding. □

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1. Morrison, S.L., Johnson, M.J., Herzenberg, L.A. & Oi, V.T. *Proc. natn. Acad. Sci. USA* **81**, 6851-6855 (1984).
2. LoBuglio, A.F. et al. *Proc. natn. Acad. Sci. USA* **86**, 4220-4224 (1989).
3. Jones, P.T., Dear, P.H., Foote, J., Neuberger, M.S. & Winter, G. *Nature* **321**, 522-525 (1986).
4. Hale, G. et al. *Lancet* **ii** **8625**, 1394-1399 (1988).
5. Buchegger, F. et al. *J. exp. Med.* **158**, 413-427 (1983).
6. Skerra, A. & Pluckthun, A. *Science* **240**, 1038-1041 (1988).
7. Riechmann, L. & Foote, J. J. *molec. Biol.* **203**, 825-828 (1988).
8. Huston, J.S. et al. *Proc. natn. Acad. Sci. USA* **85**, 5879-5883 (1988).
9. Bird, R.E. et al. *Science* **242**, 423-426 (1988).
10. Williams, W.V. et al. *Proc. natn. Acad. Sci. USA* **86**, 5537-5541 (1989).
11. Taub, R. et al. *J. biol. Chem.* **264**, 259-265 (1989).

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Biology's soft cell

Highlights at next week's American Society for Cell Biology Annual Meeting in Houston, Texas include an interactive 3-D graphics software package and formulin-free tissue fixative.

Flow Laboratories, exhibiting in booth 1241, has two new **PC-based microplate readers**, designed to scan a 96-well plate in less than two seconds (*Reader Service No. 101*). The £5,495 (UK) Multiskan Plus MKII and £6,950 (UK) Multiskan MCC/340 MKII PC systems come complete with microplate reader, IBM PC/XT or compatible, monochrome monitor, data capture and analysis software and 80-column printer. Switching assay routines is as simple as changing the disk, says Flow. The standard curve and sample results can be viewed via the monitor or printed as hard copy. The Entry Level Computing software provides statistical analyses, in-



Microplate reading IBM-compatibly. cluding mean minimum, maximum, delta, standard deviation, delta percentage and coefficient of variance for each row, column and whole plate. In addition to the five measurement modes, and nine calculation modes, the more advanced Multiskan MCC/340 MKII has a multiwavelength mode and an eight filter wheel for automatic dual wavelength reading.

In a fix

Zymed Laboratories are offering — **OmniFix — formulin-free tissue fixative** (*Reader Service No. 102*). This alcohol-based, odourless tissue fixative is, says the company, less toxic than formaldehyde and can be used with common staining techniques, such as peroxidase anti-peroxidase, avidin-biotin complex and streptavidin-peroxidase. According to Zymed, fixation times are reduced as OmniFix penetrates tissues more readily than formulin. Zymed recommends that tissues should be immersed for at least two hours. OmniFix is supplied ready-to-use in 1-, 5-, and 55-gallon polyethylene containers, and samples are available on request. Zymed will be in booth 1215.

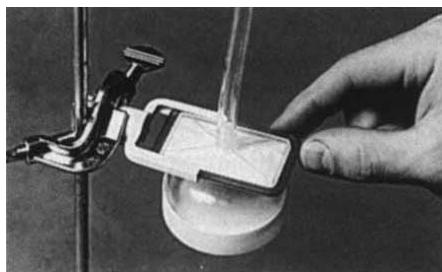
According to Vector Laboratories, their Vectabond **tissue section adhesive** can significantly increase the adherence of both frozen and paraffin-embedded tissue sections to glass slides (*Reader Service No. 103*). Vectabond chemically modifies the

glass, says Vector, to form an adherent surface that is suitable for all types of tissue sections and *in situ* hybridizations, while avoiding the instability and protease susceptibility problems associated with conventional adhesives. Vectabond costs \$35 (US) for a 7-ml concentrate that makes 350 ml of treatment solution — enough for at least 500 slides, says Vector, who will be exhibiting in booth 749.

Culture conveniences

According to Becton Dickinson Labware, increased cell production is possible with their new line of Falcon brand **expanded surface roller bottles** (*Reader Service No. 104*). The longitudinal pleat design of the roller bottles improves cell yields and reduces the amount of trypsin required for cell harvesting, says the company. Other design features of these high-grade polystyrene roller bottles include a viewing side panel and a patented plug seal cap that reduces the risk of leakage and contamination, says Becton Dickinson. The Falcon brand roller bottles are sold in cases of 20 bottles for \$144 (US). The company will be exhibiting in booths 1501/1503.

Using Gelman Sciences' **AcroCap presterilized disposable filters**, the company says serum-free culture media, media additives and buffers can be filtered at flow rates of 200 ml per minute (*Reader Service No. 105*). The AcroCap filters incorporate 15 cm² of Supor membrane, which is designed to filter up to 3 litres. The filling-bell attachment fits over wide-mouth cell culture bottles and, says Gelman, reduces the risk of contamination when filtering directly into sterile flasks. A 0.02 µm PTFE vent is incorporated into the filter's design to help prevent air blockage. The filters can attach to standard 0.25-inch tubing and can be used in conjunction with a peristaltic pump or repeating syringe. AcroCap filters come in 0.2 µm and 0.45 µm pore sizes and



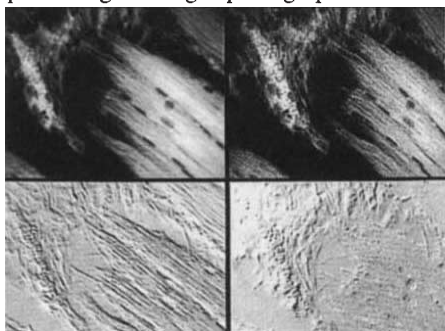
Gelman's AcroCap — visit booth 1101.

cost \$35 (US) for 10 filters. Gelman Sciences will be in booth 1101.

The right image

Visitors to booth 1042, can see VoxelView — an **interactive volume rendering software system** — from Vital Images, Inc., which allows the user to visualize and manipulate 3-dimensional data sets in real time (*Reader Service No. 106*). VoxelView graphically reconstructs objects from 3-D voxel building blocks without, says Vital Images, any loss of complexity or resolution, and at speeds of up to 4 million voxels per second. The system can render data from sources as diverse as confocal microscopes, medical imaging systems, histological sections and molecular models. Using interactive pop-up menus, a pictorial data catalogue and graphical control of rendering parameters, the user can adjust image contrast/magnification, assign pseudocolour to images, minimize artefacts, adjust lighting and transparency, rotate and view data from any angle. Objects or phenomena deemed to be short-lived can, says Vital Images, be saved as 3-D computer images. VoxelView is designed to run on any Silicon Graphics 4D/GT or GTX series workstation, and is delivered as a 0.25-inch tape cartridge complete with user's guide.

Universal Imaging Corporation, exhibiting in booths 128, 130 and 132, will be demonstrating the **Image-1/AT combined image processing and analysis system** (*Reader Service No. 107*). The \$16,900 (US) Image-1/AT Combined System runs on IBM AT-compatible computers and has menu-driven software. The video-processing board allows video-rate image processing and high-speed graphics. Other



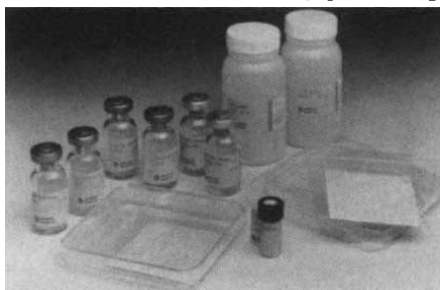
Video viewing, courtesy Universal Images.

functions include real-time image averaging with background subtraction, object counting and sizing, image sharpening, zoom and roam, 3-D image generation from optical sections, gel scanning and densitometry, text and graphics overlays. Images can be stored on magnetic disk or on an optional optical memory disk recorder. Universal emphasizes the system's built-in flexibility in that users can produce custom menus and journals — user-defined sequences of commands for repeat operations — which eliminate the need for laborious

programming and which can be recalled by a single keystroke command.

Assays, etc.

Advanced Magnetics, Inc are offering two new **immunoblot assay kits** for the measurement of phosphatidylinositol-4-monophosphate (PIP) and phosphatidylinositol 4,5-bisphosphate (PIP₂) (*Reader Service No. 108*). Samples and standards are bound to a nitrocellulose membrane and incubated overnight with monoclonal PIP antibody. The membrane is washed and incubated with goat anti-mouse IgM-HRP. The HRP substrate is developed with diaminobenzidine. Sample concentrations are determined by performing



Phosphatidylinositol phosphate kits.

a colour comparison of sample and standard dots on the membrane. Each \$395 (US) PIP and PIP₂ kit comes with sufficient reagents for 100 tests. AMI will be in booth 319.

Promega Corporation, exhibiting in booths 414/416, has **CAT reporter systems** for the direct assay of gene regulatory elements by measuring chloramphenicol acetyltransferase (CAT) activity (*Reader Service No. 109*). Genomic DNA fragments containing putative regulatory sequences are cloned into specially-constructed plasmids containing the CAT reporter gene. Four \$55 (US) pCAT plasmids are available — pCAT Basic, pCAT Enhancer, pCAT Promoter and pCAT Control — all of which share a common pUC19 backbone and express CAT activity in eukaryotic cells. Promega's CAT reporter system offers two methods for monitoring CAT enzyme activity in transfected cells: liquid scintillation counting or thin-layer chromatography. The LSC assay takes 2-3 hours and can detect 3×10^4 units (2 pg) of CAT, says Promega. The TLC assay takes longer, is less sensitive, but provides visual confirmation of assay results by autoradiography. The CAT enzyme assay, which includes 100 units chloramphenicol acetyltransferase, 25 ml Tris-HCl and 255µl *n*-butyryl-CoA, costs \$75 (US). □

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Reader Service No.37

Students for higher education

Richard Pearson

The demographic changes predicted for the 1990s may not have the effects that had been expected as there will be a rising proportion of students from non-traditional backgrounds.

EARLIER this year, the UK Secretary of State for Education and Science introduced a new era of expansion for higher education with the announcement that he wished to see a doubling of student numbers over the next 25 years. This plan has received broad support, not least from employers who see an expanding supply of good graduates as a prerequisite for a successful economy. Given the demographic downturn, whereby the number of 18-year-olds in the population will fall by a third in the decade to 1994, where will these students come from? This is the subject of a new report out this month*.

Throughout the 1960s and 1970s, higher education expanded rapidly as demand for places rose with rising attainment levels and prosperity. This growth continued through the earlier and smaller demographic downturn in the mid-1960s. In the early 1980s, this expansion showed because of the then-expected fall in demand for places resulting from the demographic downturn and the government's wish to reduce public expenditure. Nevertheless, graduate output continued to expand and in 1988 there were approximately 120,000 new first-degree graduates.

Entry to higher education continues to be dominated by young people with A-level or equivalent qualifications, who accounted for more than 80 per cent of entrants in 1988; women now account for 43 per cent of the intake. Mature students account for a slowly rising proportion of entrants, but still only about 15 per cent in 1988.

A key determinant to entry to higher education is the number of people staying on at school after the age of 16. In England and Wales, this has been increasing during the 1980s to 48 per cent in 1988. The past year has shown a significant improvement, but this figure lags well behind that achieved in Scotland. Women are more likely to stay on than men although they subsequently achieve fewer higher level qualifications. The UK staying-on rate is the ninth lowest in the European Community.

Further along the academic pipeline, A-level and equivalent attainment rates in England have improved marginally in the 1980s to total 13.8 per cent of the popula-

tion, while in Scotland they have been improving rapidly to total over 23 per cent. The proportion of these qualified young people entering higher education has reached almost 90 per cent. Overall, the percentage of the age group going on to higher education has risen slowly to reach 14.5 per cent in 1987.

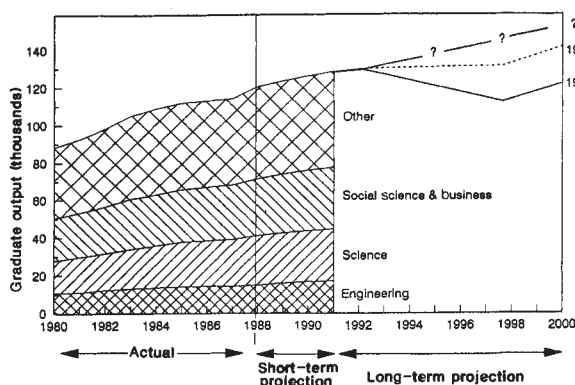
The key determinants of staying on in education, educational attainment and propensity to enter higher education are social class and parental education. In 1987, managerial and professional social classes, who formed 32 per cent of the population, accounted for 68 per cent of

business subjects (up by 31 per cent). Despite this rapid growth, engineering and technology subjects accounted for only 14 per cent of first degree output in 1988. Despite campaigns to attract them, women still accounted for only 13 per cent of the output in engineering and technology, while in education they accounted for 80 per cent and in arts and humanities 63 per cent of new graduates.

The supply of new graduates is expected to continue to increase over the period to 1992 when the demographic downturn will start to have its limited effect. The current round of educational reform in the schools

is expected to increase the inclination of students to enter higher education. A large number of access courses are also now being run to improve the ability of women, mature students and those from ethnic minorities to enter higher education.

After 1992 we now expect graduate numbers to stabilise as rising demand from women, older students, those with vocational quali-



Actual and estimated UK graduate output, 1980–2000.

entrants to the universities and slightly fewer to the public sector. This proportion has hardly changed in the past decade which suggests no widening of the intake into higher education. The importance of this factor is underlined by the fact that the demographic downturn over the decade to 1994 is concentrated in the white collar skilled and manual occupational groups, whereas the fall among the managerial and professional groups is only 10 per cent.

In the 1980s, demand for places in higher education (as expressed by university data) has grown fastest in the case of women, mature students aged over 25 (albeit from a low base) and students wishing to study business and administrative subjects; interest in engineering and technology has fallen. In terms of subject choice, women are disinclined to study engineering and applied science, while mature students are disinclined to study the applied sciences.

The fastest growth in graduations in the 1980s has been in the sciences (output up by 47 per cent), followed by engineering (up by 36 per cent) and social sciences and

cations and the young counteracts the effects of the demographic downturn. Numbers are expected to increase again at the end of the century when they will be more than 5 per cent higher than in 1989 (see figure). Within these totals, there is expected to be a rising proportion of women, of mature graduates, and of those from non-traditional backgrounds. The proportion of those with engineering and technology degrees will fall if the current swing away from these subjects is not reversed.

Thus the demographic downturn will not have the dramatic effect on higher education that had once been feared. The challenge now will be to see how numbers can be doubled, especially in the face of the introduction of student loans and increased employer competition for young people. This will be the topic of the December Employment column.

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* *How many Graduates in the 21st Century*, Pearson, R, et al, IMS, 1989.